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A Role for EHD Family Endocytic Regulators in Endothelial Biology

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A ROLE FOR EHD FAMILY ENDOCYTIC REGULATORS IN ENDOTHELIAL BIOLOGY

by

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A THESIS

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The University of Nebraska Graduate College
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Under the Supervision of Professor Hamid Band

University of Nebraska Medical Center
Omaha, Nebraska

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Advisory Committee:
Dr. Joyce Solheim
Dr. Amar Natarajan

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ABSTRACT

Endocytic trafficking is an essential process in eukaryotic cells, specifically for the transport of nutrients, membrane components, and receptors. Cargo destined for endocytic traffic is internalized at the cell surface via clathrin-dependent and clathrin-independent pathways, and brought to the early or sorting endosomes. From there, cargo is further trafficked to lysosomes for degradation, trafficked to other compartments in the cell, or recycled back to the cell surface (either directly or via the endocytic recycling compartment).

Mammalian C-terminal Eps15 homology domain-containing proteins, or EHD proteins (EHD1 to 4), are a family of highly conserved ATPases that function as key regulators of specific steps of the endocytic recycling process in metazoans. Research has shown that EHD proteins can oligomerize and bind to negatively charged membranes, which stimulates nucleotide hydrolysis. This is thought to assist in the process of membrane tubulation or the budding of vesicles, which is important in the movement of cellular components. Additionally, EHD proteins contain EH domains, which are known to bind to asparagine-proline-phenylalanine (NPF) motif-containing peptides, suggesting hundreds of potential interacting partners, albeit only a handful are thus far experimentally validated.

Recent findings have implicated EHD proteins in endothelial cells. Endothelial cells (ECs) form a barrier between blood and tissues, and play an essential role in many vital physiological functions including delivery of nutrients, angiogenesis, and innate and adaptive immune responses. Despite sharing a common purpose, ECs are structurally and functionally heterogeneous. Immunofluorescence staining of sections from multiple wild type mouse organs was carried out and the results support a conclusion of differential expression of EHD proteins within different endothelial beds. In vitro analysis of multiple cultured endothelial cell lines showed that differential EHD protein expression is lost as all four EHD proteins were expressed. Finally, siRNA-mediated knockdown of individual EHD proteins was found to affect tubulation of endothelial cells grown in reconstituted basement (Matrigel). These findings support a role for EHD proteins in endothelial cell biology.

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CHAPTER 1: INTRODUCTION

A. Endocytic Trafficking: An Overview

Endocytic trafficking requires a system of membranous compartments responsible for the sorting of internalized cargo and delivering it to various intracellular destinations. These trafficking routes are complex and provide multiple functions, contributing to important cellular processes such as receptor signaling, migration, polarity, junction formation, and membrane repair (Grant & Donaldson 2009). In the most general sense, traffic involves endocytosis of materials at the cell surface where they are delivered to the early endosome (EE) for sorting. From there, these materials can move onto the late endosome/lysosomal pathway for degradation, recycle back to the cell surface, or be brought into the *trans*-Golgi network (TGN) (Grant & Donaldson 2009) (Figure 1). Transcytosis is an offshoot of membrane traffic, utilizing the endosomal system to move cargo across a polarized cell to the opposite membrane, and will be addressed in a later section.

Regulating specific steps of endocytic traffic are the small GTP-binding Rab proteins (Smith et al. 1991). These proteins cycle between an active GTP-bound state (on) and inactive GDP-bound state (off). Conversion to the 'on' state with the assistance of guanine nucleotide exchange factors (GEFs) allows Rabs to bind and activate downstream effectors, resulting in traffic events such as vesicle formation, movement, and fusion with membranes. GTPase-activating proteins (GAPs) promote GTP hydrolysis, returning them to an off state (Smith et al. 1991). Different Rabs localize to distinct compartments and regulate specific molecular events, contributing to the high fidelity of membrane trafficking (Watson & Pessin 2006; Grant & Donaldson 2009). Rabs that are commonly associated with specific compartments and steps of trafficking will be mentioned below.

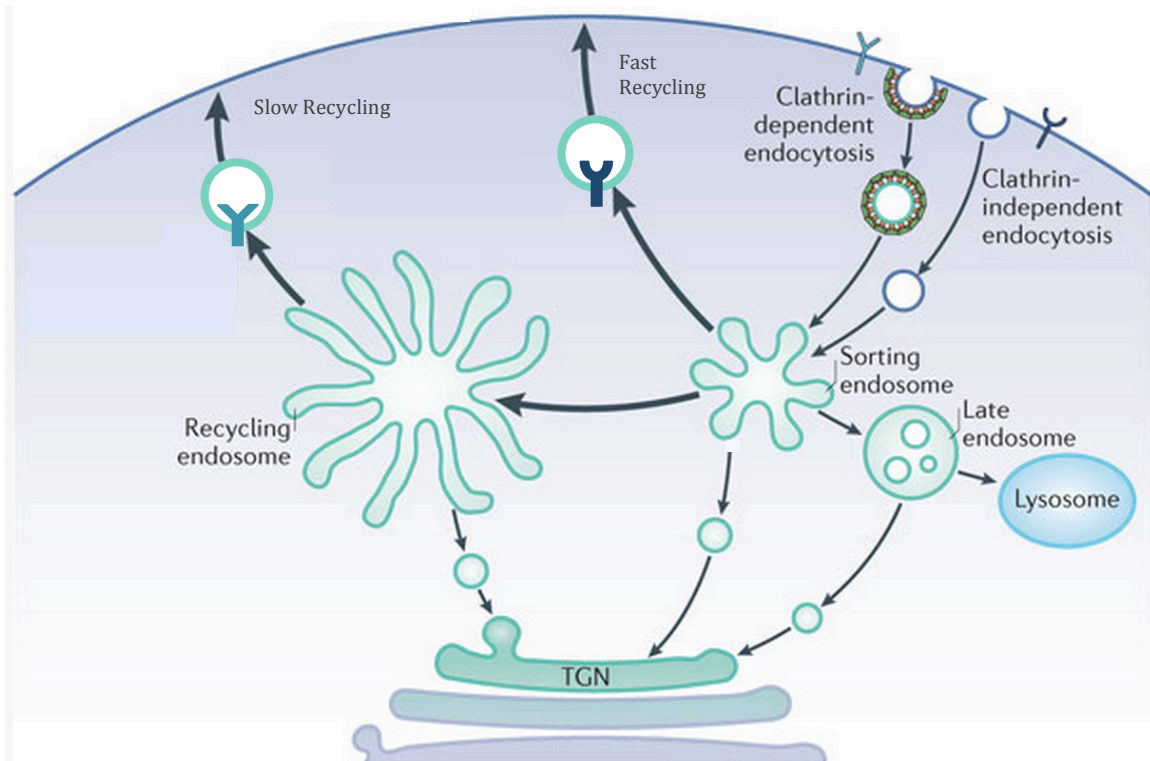


Figure 1: Model for endocytic recycling. Endocytic recycling involves the uptake of cargo via clathrin-dependent or independent methods of endocytosis followed by sorting in the early, or sorting, endosome. Cargo is ultimately either recycled back to the cell surface via fast and slow recycling mechanisms or is degraded in the lysosome. Additionally, cargo may be trafficked to and from the *trans*-Golgi network via retrograde transport.

Adapted from Hsu, V. W. et al. Getting active: protein sorting in endocytic recycling. *Nature Reviews Molecular Cell Biology*, 3(5) 2012.

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Cellular cargo is first internalized via endocytosis. Endocytic events can be subdivided into clathrin-dependent (CDE) and clathrin-independent endocytosis (CIE) (Conner & Schmid 2003; Doherty & McMahon 2009). CDE, the best-studied pathway, begins with the adapter protein AP-2 recognizing and binding the intracellular domain of a protein, bridging the cargo to the scaffold protein clathrin. A clathrin-coated pit is formed, and the GTPase dynamin regulates the fission of the budding vesicle from the plasma membrane (Hinshaw 2000; Maxfield & McGraw 2004). Once the vesicle has

entered the cell, the clathrin coat and adaptor proteins are removed and recycled (Maxfield & McGraw 2004). Receptor-mediated endocytosis commonly utilizes this route, and transferrin receptor (TfR) and low density lipoprotein (LDLR) are well-known cargoes of CDE (Grant & Donaldson 2009).

Many cell surface proteins lack the intracellular domain required for CDE, and, therefore, can be internalized via one of several clathrin-independent pathways. CIE pathways can be categorized based on their dependence on dynamin (Mayor & Pagano 2007). Caveolae-mediated endocytosis is dynamin dependent and well characterized. Caveolae are invaginations that are characterized by their flask-shaped invaginations and the presence of caveolin (Parton 2003), and that are enriched with sphingolipids and cholesterol (Aboulaich et al. 2004; Lemaître et al. 2005; Sprenger et al. 2004). Caveolae carry a variety of cargos including receptors, lipids, and pathogens (Mayor & Pagano 2007). Another dynamin-dependent CIE pathway involves RhoA-mediated internalization via detergent-resistant membranes (Mayor & Pagano 2007; Lamaze et al. 2001). One form of dynamin-independent CIE requires the GTPase ARF6. This pathway has been identified as the internalization route for MHC class I molecule, β 1-integrin, and E-cadherin (Radhakrishna & Donaldson 1997; Walseng et al. 2008; Brown et al. 2001; Powelka et al. 2004). Finally, specialized forms of CIE involve actin-driven events and include phagocytosis and macro-pinocytosis (Sigismund et al. 2012). This thesis does not provide a comprehensive list of all endocytosis pathways that have been identified, and it is important to point out that these pathways are complex and there is evidence of crosstalk between them.

Once internalized, most endocytosed material is delivered to the early endosome (EE) via intermediate vesicles or tubules and then sorted (Mayor & Pagano 2007). EEs are tubular-vesicular compartments located near the periphery of the cell. Rab5, EEA1,

and PI3K serve as markers for EEs and are important in their function (Grant & Donaldson 2009). From the EE, endocytic cargo can travel back to the cell surface, continue on to the endocytic recycling compartment (ERC), or remain in the EE as it matures and moves towards degradation in the lysosomes. Cargo that recycles to the plasma membrane leaves the EE rapidly in vesicles that pinch off from tubules (Grant & Donaldson 2009), a process regulated by Rab4. EEs are slightly acidic, promoting the dissociation of ligands from receptors. The EE then moves along microtubules further into the cell, acquiring hydrolases that drop the pH further as part of the maturation process to late endosome (LE). Cargo is then delivered to the lysosomes for degradation. Proteins fated for the lysosome are modified by the addition of ubiquitin to their lysine residues, serving as a 'termination signal' (Maxfield & McGraw 2004).

Cargo that isn't rapidly recycled or degraded moves onto the ERC via extension of tubules from the EE that lose Rab5 and acquire Rab11 (Grant & Donaldson 2009). The ERC is usually located at microtubule-organizing centers and consist of long-lived organelles. While there are several fates for cargo within the ERC, most of the cargo recycles to the cell surface via the slow recycling pathway. Movement between the ERC and *trans*-Golgi network can occur as well (Maxfield & McGraw 2004). Interestingly, in some instances receptors can be trafficked from the ERC to specialized compartments, such as the case with GLUT4. GLUT4 is stored in insulin-regulated compartments (IRCs) and upon stimulation with insulin GLUT4 is recycled to the plasma membrane from this compartment. This process is dynamic, and GLUT4 is equally present in the ERC (Zeigerer et al. 2002; Lampson et al. 2001; Maxfield & McGraw 2004).

B. EHD Proteins: Regulators of Endocytic Traffic

One subset of proteins involved in endocytic trafficking events is characterized by multiple Eps15 homology (EH) domains, which were first identified as three repeated copies at the N-terminus of epidermal growth factor receptor tyrosine kinase substrate Eps15 (Fazioli et al. 1993; Wong et al. 1994). Since then, many EH domain-containing proteins have been implicated in early endocytic events (Confalonieri & Di Fiore 2002; Santolini et al. 1999). The EH domain is comprised of approximately 100 highly conserved amino acids (Fazioli et al. 1993; Wong et al. 1994). Structurally, the EH domain forms a fold containing two EF-hands, or helix-loop-helix motifs, connected by a short antiparallel β -sheet. These EF-hands are also capable of binding calcium (de Beer et al. 1998; Confalonieri & Di Fiore 2002). One of the functions of the EH domain is to recognize asparagine-proline-guanine (NPF) motifs (Santolini et al. 1999; Polo et al. 2003; Salcini et al. 1997). This interaction occurs within a hydrophobic pocket found on the surface of the EH domain, facilitating a close proximity between a highly conserved tryptophan and the asparagine of the NPF motif (de Beer et al. 1998; de Beer et al. 2000; Rumpf et al. 2008).

A subfamily of the EH-domain containing proteins, the C-terminal Eps15 homology domain-containing proteins (EHD1-4), is unique in that these proteins contain a single C-terminal EH domain. EHD proteins are conserved amongst different species. Some, such as *C. elegans* and *D. melanogaster* have a single ortholog of human EHD1 (Rme-1 and Past1 respectively) (Grant et al. 2001; Olswang-Kutz et al. 2009). Initial studies in *C. elegans* found that mutations in RME-1 led to defects in endocytic trafficking (Grant et al. 2001). Additionally, EHDs have been linked to Rab proteins, their effectors, and recycling of receptors, supporting their importance in endocytic trafficking events (discussed below).

The EHD proteins are encoded by four different genes that are located on separate chromosomes (Pohl et al. 2000). Despite this, EHD proteins are highly conserved, sharing an overall amino acid sequence identity of 71-86% (Pohl et al. 2000). They are approximately 60 kDa in mass and are characterized as having three major functional domains (Figure 2). The C-terminal EH domain facilitates protein-protein interactions. The N-terminal G-domain, or P-loop, is a nucleotide-binding site that preferentially hydrolyzes ATP, instead of GTP, which is preferentially used by GTPases such as Rabs and dynamin. Finally, the central helical (or coiled-coiled domain) mediates homo- and hetero-oligomerization. It has been proposed that EHD proteins function in membrane fission through dimerization and lipid binding, powered by the hydrolysis of ATP (Daumke et al. 2007). Previous studies have suggested roles for EHD proteins in membrane bending, tubulation, and vesiculation (Pant et al. 2009; Daumke et al. 2007; Jakobsson et al. 2011; Cai et al. 2012). A very recent study has provided a clearer image of their functions, suggesting that EHD1 and 4 are vesiculators and EHD3 is a tubulating protein (Cai et al. 2013).

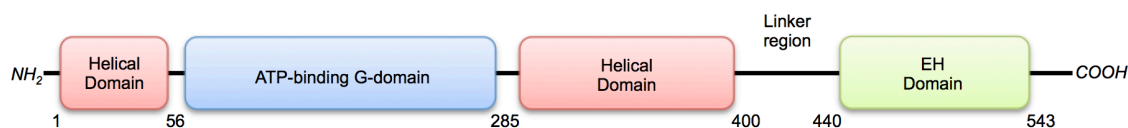


Figure 2: EHD protein architecture. EHD proteins have 3 characteristic domains. EHD proteins differ from other EH domain-containing proteins in that they contain a single EH domain at the C-terminus. The EH domain recognizes and binds other proteins that contain NPF motifs. The N-terminus contains an ATP-binding G-domain, or P-loop that binds nucleotides. Finally, there are two helical domains that are important for oligomerization.

EHD1 is the most extensively studied of the EHD proteins, most likely because of its sequence homology to orthologs found in other species. A prominent function for EHD1 is the regulation of receptor recycling from the endocytic recycling compartment to the plasma membrane, including receptors that have been internalized via both CIE and CDE. Receptors endocytosed via CDE associated with EHD1 include Transferrin receptor (TfR) (Mintz et al. 1999; Lin et al. 2001), GLUT4 glucose transporter (which can also internalize via CIE) (Guilherme, Soriano, Furcinitti, et al. 2004), and AMPA receptor (Park et al. 2004; Carroll et al. 2001). Receptors endocytosed via CIE include MHC class I and II molecules (Caplan et al. 2002; Walseng et al. 2008), β -1 integrin (Jović et al. 2007), and cystic fibrosis transmembrane conductance regulator (CFTR) (Picciano et al. 2003; Holleran et al. 2013). In respect to GLUT4 recycling, EHD1 interacts with EHBP1, which is required for mobilization of GLUT4 upon stimulation with insulin in adipocytes (Guilherme, Soriano, Furcinitti, et al. 2004). Additionally, EHD1 associates with other proteins important in the recycling step of endocytic trafficking including Rabenosyn-5 (an effector of Rab4/Rab5) (Naslavsky et al. 2004), and Syndapin I and II (Xu et al.; Braun et al. 2005).

In addition to trafficking receptors from the ERC to the plasma membrane, there is evidence that EHD1 is involved in transport from the EE to the ERC. This is supported by EHD1/3's interaction with Rab11-FIP2 (Naslavsky 2005). EHD1 is also associated with Rab35, which forms a complex with MICAL-L1 and other proteins to promote EE to ERC transport (Kobayashi & Fukuda 2013). EHD1 has been implicated in endocytic events, including the internalization of low-density lipoprotein (LDL) receptor (Naslavsky et al. 2007), L1/neuron-glia cell adhesion molecule (NgCAM) along with EHD4 (Yap et al. 2010), and IGF1R (Rotem-Yehudar et al. 2001). Finally, EHD1 is involved with retrograde transport in association with the retromer complex (Gokool et al. 2007).

More recently, research has revealed novel roles for EHD1 in cell biology. Along with MICAL-L1, EHD1 is involved in microtubule alignment during mitosis, assisting in chromosome alignment and cytokinesis (Reinecke et al. 2015). Additionally, along with EHD3, EHD1 localizes to the ciliary pocket and is indispensable for early assembly events of cilia (Lu et al. 2015).

In vivo studies utilizing *Ehd1*-knockout mice revealed defects in spermatogenesis, muscle fibers, and ocular development. *Ehd1*-null mice are born at sub-Mendelian ratios and are smaller at birth. Additionally, males are infertile and testes are smaller. This is due to a disruption in the spermatogenic cycle that results in the absence of mature spermatozoa (Rainey et al. 2010a). *Ehd1* deletion also leads to smaller muscle fibers, most likely as a result of reduced myoblast fusion (Posey et al. 2014). Finally, the most recent study done in *Ehd1*-knockout mice shows that approximately 50% are born with some form of ocular deformity including small/absent lenses and cataracts. Defects in epithelial proliferation and survival and loss of cellular junction expression contributed to this phenotype (Arya et al. 2015).

EHD2 is the first and only EHD protein to have its complete structure solved (Daumke et al. 2007). EHD2 has been implicated in the regulation of few receptors, but it has obvious importance in endocytic trafficking. EHD2 binds EHBP1 and regulates the internalization of transferrin receptor and GLUT4, both internalized via clathrin-mediated endocytosis (Guilherme, Soriano, Bose, et al. 2004). On the other hand, EHD has been implicated in the recycling of transferrin receptor from the ERC as well (George et al. 2007). EHD2 has also been linked to clathrin-independent endocytosis, specifically caveolae mediated. EHD2 forms oligomers that localize to caveolae at the plasma membrane in an ATP dependent process (Stoeber et al. 2012). Additionally, EHD2 has been found to complex with pacsin2, a caveolaer protein (Hansen et al. 2011; Ludwig et

al. 2013). Along with EHD1, EHD2 also interacts with Fer1L5 as well as another ferlin family protein, myoferlin, and depletion of EHD2 reduces myoblast fusion (Posey et al. 2011; Doherty et al. 2008). Laser-induced injury of muscle fibers causes an accumulation of fluorescently labeled EHD2 to the injury site in a dome shape where a third ferlin family member, dysferlin, localizes (Marg et al. 2012). This suggests a novel role for EHD proteins in plasma membrane repair. Finally, in a function unrelated to cellular traffic, EHD2 was recently found to shuttle to the nucleus where it can repress transcription of cyclin-dependent kinase inhibitor 1 (CDKN1A) (Pekar et al. 2012).

EHD3 is most similar in sequence to, and directly binds to, EHD1 (Galperin et al. 2002). EHD3 functions differently, though, in that it is primarily involved in early recycling events, specifically, EE to ERC transport (Galperin et al. 2002; George et al. 2007). EHD3 has also been associated with early endosome to Golgi retrograde transport (Naslavsky et al. 2009). More recently, EHD3 was found to play a role in the rapid recycling of the $\text{Av}\beta 3$ -integrin from the early endosome to plasma membrane, playing an important role in cell survival and adhesion (Waxmonsky & Conner 2013). Like EHD2, EHD3 undergoes post-translational modification via SUMOylation. In the case with EHD3, SUMOylation at Lysine residues 315 and 511 are involved in the tubulation of the endocytic recycling compartment and necessary are for the successful recycling of transferrin receptor to the cell surface (Cabasso et al. 2015). The most extensive studies involving EHD3 have been in the mouse heart. EHD3 was found to interact with Ankyrin-B, regulating the sodium/calcium exchanger (NCX1) in cardiomyocytes (Gudmundsson et al. 2010). In multiple mammalian heart failure models, EHD3 along with NCX1 was upregulated, suggesting EHD3 is an important component to cardiac remodeling post injury (Gudmundsson et al. 2012). Later the same group implicated EHD3 in the trafficking of L-type calcium channel type 1.2 (Cav1.2) in myocytes and voltage gated T-

type calcium channels 3.1 and 3.2 (Cav3.1/Cav3.2) in atrial myocytes (Curran et al. 2014; Curran et al. 2015).

EHD4, which is also called pincher, has primarily been identified in transport from the early endosome to the endocytic recycling compartment or the lysosomal pathway (George et al. 2007; Sharma et al. 2008). It was first found to interact with collagen type VI as an extracellular protein (Kuo 2001). *In vivo*, male *Ehd4* knockout mice display fifty percent smaller testis size and are sub-fertile, suggesting a role for EHD4 in germ cell development. While knockout mice show no evidence of hearing disability, EHD4 interacts with and regulates cadherin 23, a protein important for delivering mechanical signals in the inner ear (Sengupta et al. 2009).

EHD4 expression is prominent in the brain, and in neuronal cells EHD4 is involved in the trafficking in several receptors. Despite this, there are no neurological phenotypes reported in *Ehd4*-knockout mice to date. EHD4 is linked to pinocytic endocytosis of the nerve growth factor receptors TrkA and TrkB, promoting their signaling (Shao et al. 2002; Valdez et al. 2005; Philippidou et al. 2011). Nogo-A is a protein responsible for axonal growth inhibition, and its internalization is also regulated by EHD4 (Joset et al. 2010). EHD4 oligomerizes with EHD1, and together they regulate the internalization of the Ln/NgCAM in neurons as well. In *Drosophila*, NUMB is neurological protein important in cell fate and it interacts with EHD4.

One final note about EHD proteins is their ability to compensate for one another. All 4 mammalian EHD proteins are capable of rescuing the function of Rme-1 when defective in *C. elegans* (George et al. 2007). As discussed above, though, it is apparent that each EHD protein has a distinct function. Additionally, single deletion of an *Ehd* gene in mice has resulted in increased expression of other EHD proteins. For example, EHD1 is upregulated in the ear and testes when *Ehd4* is deleted (Sengupta et al. 2009;

George et al. 2010). Also, EHD4 is upregulated in glomerular endothelial cells in the kidney of *Ehd3*-null mice (discussed below) (George et al. 2011).

C. Localization of EHD Proteins in Endothelial Beds

Only a handful of journal articles address EHD protein expression within endothelial beds. Transcriptome analysis show enrichment of *ehd1* expression in endothelial cells from whole organs, most significantly in the lung, from Tie2GFP mice (Daneman et al. 2010). Tie2GFP mice have endothelial cell-specific expression of GFP. When isolated and co-cultured with glial cells to recreate the blood-brain barrier environment, bovine brain capillary endothelial cells increase expression of *Ehd1* mRNA and protein compared to cells that have not been co-cultured (Deracinois et al. 2012). Through immunofluorescent staining of mouse tissue sections, EHD2 has been found to localize within vasculature of skeletal muscle and interlobular arteries of kidneys (Mate et al. 2012; George et al. 2011). Immunofluorescent staining of liver sections from rats showed that EHD3 co-localizes with the scavenger receptor Stabilin-1 in sinusoids (Géraud et al. 2010). A study involving hepatocellular carcinoma in a rat tumor model found that EHD3 was heavily down regulated in tumor endothelial cells. Further analysis using IHC found that EHD3 was expressed in sinusoidal endothelial cells of the normal liver tissue and expression was lost in the endothelium of adjacent tumor endothelium (Jia et al. 2010). EHD3 has also been found in glomerular endothelia of the mouse kidney via microarray data, northern blot, western blot, and immunofluorescent staining analysis (Patrakka et al. 2007; Brunskill & Potter 2010; George et al. 2011). Just from these few articles, it's apparent that EHDs are expressed in the endothelium and perhaps are expressed differentially in different endothelial bed types. Additionally, changes seen in the vasculature during cancer development may affect EHD protein expression as well.

Previous studies show when a single EHD protein gene is deleted, there are compensatory changes in other EHD protein's expression (Rainey et al. 2010b; George

et al. 2010; Sengupta et al. 2009). In *Ehd3* knockout mice, EHD4 is up regulated in glomerular endothelial cells. Concurrent deletion of *Ehd3* and *Ehd4* led to an increase in EHD2 in the glomerular and peritubular capillary endothelium (George et al. 2011). Interestingly, removing the compensation of EHD4 in the double knockout mice also caused thrombotic microangiopathy (TMA), a pathology of the endothelia commonly seen in conditions such as pre-eclampsia and malignant hypertension (Stillman & Karumanchi 2007). Additionally, this phenotype is also seen in mice when podocyte-expressed VEGF-A is knocked down in mice (Eremina et al. 2008; Eremina et al. 2003). Subsequently, *Ehd3*^{-/-}; *Ehd4*^{-/-} glomeruli show a more dispersed distribution of VEGFR2 (George et al. 2011). This is the first evidence that EHD proteins may be playing a role in the trafficking of a receptor (VEGFR2) in endothelial cells.

D. Vascular Endothelial Cells and Their Heterogeneity

In 1628 William Harvey first described a closed circulatory system that consisted of arteries and veins while predicting the existence of capillary beds (Aird 2007a). The purpose of this closed circulatory, or cardiovascular, system is to deliver nutrients and oxygen to the tissues of the body. This is accomplished when blood is pumped away from the heart through the arteries, carrying oxygen and nutrients and delivering them to tissues and cells via the capillary network. This same network also picks up carbon dioxide and waste, and the blood makes its way back towards the heart through the veins. Both arteries and veins are made up of three layers: *tunica intima* (endothelial cells), *tunica media* (smooth muscle and connective tissue), and *tunica adventitia* (connective tissue). Capillaries differ from arteries and veins in that they consist of just a layer of endothelium and sometimes connective tissue. Comparing arteries to veins, arteries have a thick *tunica media* and pulsate while veins have thin walls and valves (Aird 2007b).

Wilhelm His was the first to define the term endothelium in 1865 (Aird 2007a). Endothelial cells (ECs) make up the inner lining of both blood vessels and lymphatics, covering an approximate area of 1 to 7 m² and accounting for 60 trillion cells found in the body (Augustin et al. 1994). ECs are the gatekeepers of the cardiovascular system, playing a part in many vital physiological functions including delivery of nutrients, angiogenesis, and innate and adaptive immune responses. The endothelium is implicated in most, if not all, human diseases and provides immense therapeutic potential.

The heterogeneous nature of ECs first became obvious when studies using electron microscopy (EM) in the 60s led to the discovery that there were many types and that they differed in structure (Florey 1966). While ECs share the same general purpose

of maintaining a barrier between blood and tissue, their phenotypes vary based on factors such as their location, endothelial bed type, structure, and function.

ECs vary structurally based on their location. Their thickness can range from less than 0.1 μm in the smallest capillary ECs to 1 μm in the aorta. Blood flow and shear stress dictate the shape of ECs. In general ECs from arteries are long and narrow (spindle shaped), ECs from veins are more short and broad (rectangular), and capillary ECs are irregularly shaped (Aird 2007a). Interestingly, while most ECs are flat, high endothelial (post-capillary) venules are plump and cuboidal. This is most likely to accommodate leukocyte migration (Girard & Springer 1995). Intercellular junctions are formed between all endothelial cells and include tight and adherens junctions (Dejana 2004; Bazzoni & Dejana 2004). Endothelial cells found in large arteries contain many tight junctions due to the high exposure to shear stress. Capillaries vary in number of tight junctions, with the least amount found in the post-capillary venules, again, due to their function in leukocyte migration. The blood brain barrier, on the other hand, has a high number of tight junctions in order to protect neural tissues (Aird 2007a).

Endothelial beds can be categorized into three subsets: continuous nonfenestrated, continuous fenestrated, and discontinuous/sinusoidal. Continuous nonfenestrated endothelia have an uninterrupted basement membrane, and their ECs are tightly connected. These endothelial beds are found in all arteries and veins in addition to some capillary beds. Continuous fenestrated endothelia also have a continuous basement membrane, but cells are interrupted with transcellular pores, or fenestrae, that are approximately 70 nm in diameter. In most cases, they contain a thin diaphragm, contributing to permeability. Fenestrated endothelia function as filters and are found in capillary beds of the kidney (glomerulus and some renal tubules), intestinal mucosa, and endocrine glands. Fenestrated endothelia can be found in other vessels

during development, but are lost over time (Yoshida et al. 1988; Stewart & Hayakawa 1994). Discontinuous endothelial beds are characterized by their poorly formed basement membrane in addition to the presence of large fenestrae (100-200 nm in diameter) and large gaps. These endothelia are most commonly associated with the sinusoids of the liver (Aird 2007a). Figure 3 provides a summary of the characteristics of ECs and the endothelial beds they make up in different blood vessel types.

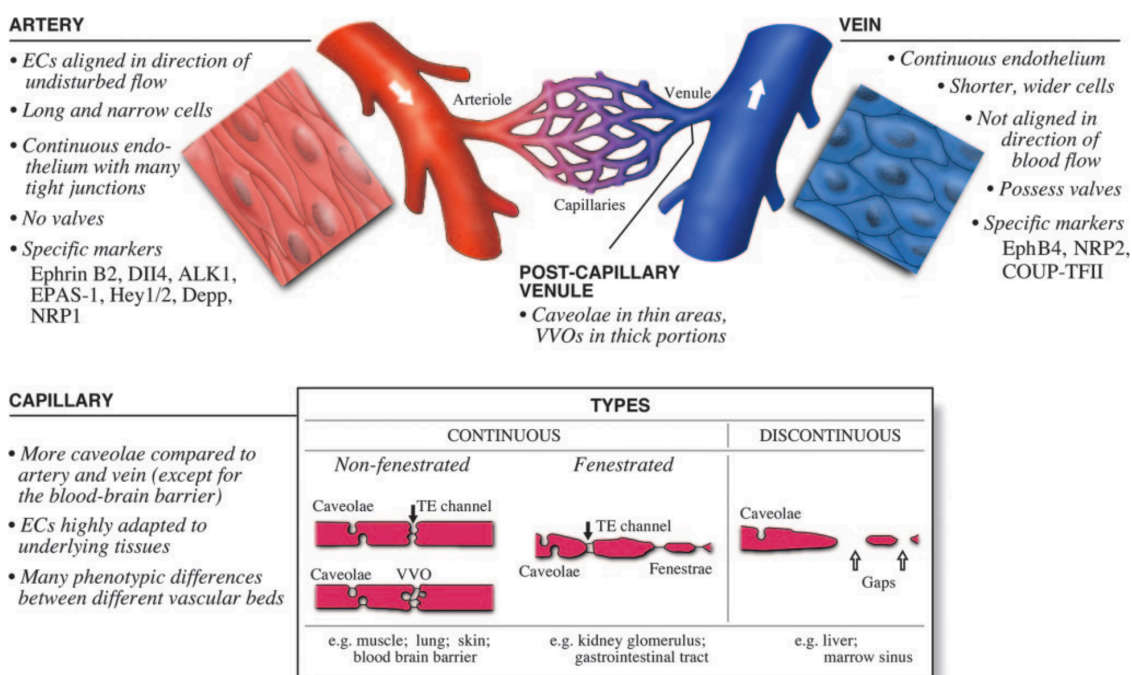


Figure 3: Endothelial cells of arteries, veins, and capillaries. Endothelial cells vary depending on the blood vessels they line. Structurally they vary between artery, vein, and capillary bed. Arteries and veins are composed of continuous endothelial cells. Capillaries can further be broken down into continuous, fenestrated, and discontinuous and are found in different tissues and organs.

From: Aird, W.C., 2007b. Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. *Circulation research*, 100(2), pp.174–90.

Both the structure of endothelial cells and the organization of endothelial beds are dynamic and can be altered. Experiments have shown that reorienting ECs will cause them to realign with the direction of blood flow (Flaherty et al. 1972). Both fenestrated and sinusoidal endothelia formation and maintenance are dependent on VEGF signaling. Loss of VEGF can result in the loss of fenestrated endothelia (Maynard et al. 2003; Eremina et al. 2008; Carpenter et al. 2005). Stimulation of VEGF, on the other hand, can result in induction of fenestrae in continuous endothelium (Roberts & Palade 1995). Disease can also result in disruption of endothelial beds. For example, liver disease can result in loss of sinusoids and gain of continuous endothelium (Bhunchet & Fujieda 1993).

Endothelia also display functional heterogeneity based on blood vessel type and location. One function of the endothelium is to be permeable. Capillaries are basally permeable in that there is continuous movement of material between the blood and tissues. Post-capillary venules, in contrast, become permeable when induced by an agonist – usually as an inflammatory response. Additionally, the post-capillary venule is also the site of leukocyte transmigration, which is facilitated by adhesion molecules such as P- and E-selectins. Another function of endothelium is to maintain homeostasis, including maintenance of the blood by expressing coagulants and anticoagulants, all of which are expressed differentially in different blood vessels. In addition to these functions, different endothelial beds have specialized functions. Examples include filtration in glomerular endothelial cells of the kidney and sinusoids of the liver. The sinusoids in the liver are also responsible for scavenging and immune tolerance. Finally, endothelial cells are generally considered quiescent. Endothelium in reproductive organs in females, though, undergoes high levels of proliferation and angiogenesis (Aird 2007a). Table 1 summarizes the different levels of heterogeneity seen in endothelial cells.

STRUCTURE	EXPRESSION PATTERNS	FUNCTION
Size and shape	Protein	Hemostasis
Nuclear orientation	mRNA	Vasomotor tone
Thickness	Transcription networks	Barrier function
Microvilli	Signaling pathways	Leukocyte trafficking
Filaments		Cell survival
Vesicles		Cell migration
Junctions		Cell proliferation
		Antigen presentation

Table 1: Endothelial cell heterogeneity. Endothelial cells vary by structure, expression patterns, and function. These traits are greatly affected by their location, such as blood vessel or organ (Aird 2003).

In general, due to their heterogeneity, identifying ECs is a difficult task. For example, ultrastructural features such as caveolae and Weibel-Palade bodies can be used to identify most endothelia types through microscopy, but caveolae are commonly found in many other cell types and not all ECs have Weibel-Palade bodies (Weibel & Palade 1964). Additionally, gene expression in ECs is differential throughout the entire vasculature, making it hard to identify true markers of endothelial cells (Aird 2003). Table 2 lists common markers used for endothelial cells. Other markers have been used for specialized vasculature as well, including Glut-1 for blood brain barrier endothelium and GlyCAM-1 for high endothelial venules (Garlanda & Dejana 1997). It is important to point out that in addition to uneven expression of these markers, some of these molecules are also expressed in non-endothelial cell types. Currently, the most commonly used markers to encompass most endothelial cells are platelet/endothelial cell adhesion molecule (PECAM)-1 (or CD31) and vascular endothelial (VE)-cadherin (Aird 2007a; Garlanda & Dejana 1997).

MARKER	SPECIES
Angiotensin-converting Enzyme	Human/Mouse
AAMP	Human
CD102/ICAM-2	Human/Mouse
CD105/endoglin	Human
CD31/PECAM-1	Human/Mouse
CD34	Human/Mouse
*CD36	Human
CD51/CD61 (Vitronectin receptor)	Human/Mouse
CD73/VAP-2	Human
Factor VIII-related antigen	Human/Mouse
*HEMCAM	Mouse
<i>Griffonia simplicifolia</i> lectin I	Mouse
Sca-1	Mouse
S-ENDO 1/MUC18	Human/Mouse
Thrombomodulin	Human/Mouse
Type I scavenger receptor (acetylated-LDL uptake)	Human/Mouse
<i>Ulex europaeus</i>	Human

Table 2: Endothelial specific markers. The above table lists known markers for endothelial cells in both humans and mice. Asterisks note markers for microvascular endothelial cells. These proteins/markers can identify other cell types as well (Garlanda & Dejana 1997). Table adapted from Garlanda & Dejana 2007.

E. Endocytic Trafficking in Endothelial Cells

Just like any other cell, endothelial cells are capable of trafficking cellular cargo. In addition to conventional endocytic trafficking (as described above), ECs also carry out transcytosis to deliver materials to underlying tissues. While transcytosis is seen in other polarized cell types, it was first identified within capillaries in the 1950s (Tuma & Hubbard 2003). Transcellular transport involves the movement of materials from one side to the other through the cell as opposed to between adjacent cells, which is seen in paracellular transport. Only continuous and fenestrated endothelium participate in transcytosis and capillaries are most actively utilizing transcytosis (Simionescu 1983; Simionescu & Simionescu 1991). Movement of cargo can occur in both directions (blood to tissue and vice versa) and by both bulk- and receptor-mediated mechanisms (Tuma & Hubbard 2003).

While ECs contain the machinery of endocytic recycling, it is less abundant and more localized at the perinuclear region of the cell. Unlike other cell types, CIE is not the prevalent route for internalization seen in ECs. The population of coated pits in ECs tends to be significantly lower than that of non-coated vesicles. TfR and LDLR are traditionally internalized via CIE, and this holds true in ECs (Muro et al. 2004). Additionally, recycling of the inducible adhesion molecules E- and P-selectin is regulated by clathrin-mediated uptake (von Asmuth et al. 1992; Straley & Green 2000). Most notably, ECs of the liver sinusoids rely heavily on CIE for the internalization of IgG immune complexes by *Fe* receptors (Kosugi et al. 1992).

Caveolae-dependent endocytosis is more prominent in ECs and is also utilized as the entry point for transcytosis (Muro et al. 2004). In addition to contributing to internalization, caveolae contain the components required for vesicle formation, fission, docking, and fusion, allowing for involvement in other physiological functions (Schnitzer

et al. 1995). Indeed, caveolae have now been implicated in many EC functions such as permeability, mechanotransduction, and redox signaling (Sowa 2012). Another area in which caveolae have been implicated is during angiogenesis. Caveolae merge and form large structures during angiogenesis; overexpression of caveolin-1 enhanced caveolae formation, leading to increased tubule formation in ECs (Esser et al. 1998; Liu et al. 2002). Additionally, VEGF receptor (VEGFR2), whose signaling is required for angiogenesis, localizes to caveolae. Given previous studies associating EHD2 with caveolae, EHD proteins may play a role in endothelial trafficking and function as well.

F. Hypothesis

Endothelial cells utilize endocytic trafficking for many cellular processes including membrane repair, angiogenesis, and receptor trafficking. Previous publications already show that EHD proteins, which are key regulators in trafficking, are expressed in endothelial beds of multiple organs. Given the heterogeneous nature of ECs as well as the distinct roles of different EHD proteins, it is hypothesized that EHD proteins will be expressed differentially based on endothelial bed type. Based on previous data, we predict that EHD2 is expressed in continuous nonfenestrated endothelium while EHD3 is expressed in continuous fenestrated and sinusoidal capillary beds. EHD1 may be expressed in endothelial beds where tight junctions are more prevalent, such as arteries. Additionally, we predict that EHD proteins will be also expressed in endothelial cell lines, and that knockdown of EHD proteins *in vitro* will disrupt expression of various endothelial receptors and affect tubulation.

To test these hypotheses and to begin to understand the role of EHD proteins in endothelial cell biology, the following will be accomplished:

1. Determine EHD expression throughout different endothelial beds of wild type mouse organs via immunofluorescent staining analysis.
2. Determine EHD expression in endothelial cell lines via western blot analysis and examine whether expression varies between different cell lines.
3. Assess the effects of siRNA-mediated knockdown of EHD1 and EHD2 *in vitro* on VEGFA-stimulated tubulation of endothelial cells in growth factor-reduced Matrigel.

CHAPTER 2: METHODS AND METHODS

A. Tissue Staining

The following organs were harvested from perfused wild type (C57Bl6) mice: kidney, heart, lungs, liver, and pancreas. Organs were formalin fixed overnight, embedded in paraffin, and sectioned (5 μ m) for immunofluorescent staining. Sections were deparaffinized in xylene and rehydrated in graded ethanols followed by a PBS wash. Slides were then boiled in a citrate-based antigen unmasking solution (Vector Laboratories) in a large flask in the microwave followed by three washes in water and one wash in PBS. Sections were blocked in PBS/5% fetal bovine serum (FBS) for 1 hour. Rabbit monoclonal anti-EHD1 primary antibody (Abcam) was commercially purchased and used at a dilution of 1:500 in PBS/5% FBS. Polyclonal rabbit anti-EHD2 and EHD3 primary antibodies were used at a 1:200 dilution. Fluorescein-labeled tomato lectin (from *L. esculentum*) and GSL 1 – isolectin B4 (from *G. simplicifolia*) were used at 1:250 and 1:50 dilutions, respectively (Vector Labs). Donkey anti-rabbit Alexa Fluor 594 secondary antibody was used at 1:500 dilution (Invitrogen). Slides were mounted in Vectashield containing DAPI and sealed with clear nail polish. Images were acquired with EVOS FL Auto microscope (Life Technologies).

B. Cell Culture

Human umbilical vein endothelial cells (HUVECs) were grown in Media 200 supplemented with low serum growth supplement (LSGS) (ThermoFisher Scientific). EA.Hy926 cells (ATCC) were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100 units/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). Human microvascular endothelial cells (HMEC1) were provided by Dr. Rakesh Singh's lab (University of Nebraska Medical Center) and were grown in RPMI-1640 Medium

containing 5% FBS, L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Mouse mammary fat pad microvascular endothelial cells (MFP MVECs) were also provided by Dr. Rakesh Singh's lab and maintained in flasks coated with 0.2% gelatin. MFP MVECs were maintained in DMEM containing 10% FBS, 2 mM GlutaMAX (Gibco), 1 mM sodium pyruvate (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 1 X MEM vitamins (MediaTech), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen), 0.25 µg/ml amphotericin B (Fungizone) (ThermoFisher Scientific), and 40 µg/ml gentamicin (Invitrogen). MFP MVECs were isolated from H-2Kb-ts-A58 mice (Immortomice from Charles River Laboratories), and express SV40 antigen at 33°C and lose SV40 expression at 37°C. All cell lines were passaged using trypsin-EDTA solution, except for MFP-MVECs, which required Accutase dissociation solution (Innovative Cell Technologies). All cells were grown in an atmosphere of 95% air and 5% CO₂.

C. Antibodies and Western Blotting

Cells were lysed in 10% Triton X cell lysis buffer (1 M Tris 7.5, 5 M NaCl, 10% Triton X-100, 100 mM VO₄, 1 M NaF, 50 nM PMSF) overnight at 4°C. Lysates were clarified and protein concentration was determined in a 96 well plate using the Bio-Rad D_c Protein Assay, using bovine serum albumin as a standard. Aliquots containing 40 µg of protein were prepared in sample buffer and separated using 7.5% SDS-polyacrylamide gel electrophoresis (PAGE), then the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were immunoblotted with appropriate primary antibodies followed by 1:20,000 dilutions of horseradish peroxidase (HRP)-conjugated protein A, Goat anti-Rabbit, or Goat anti-mouse secondary antibodies. Signals were detected using enhanced chemiluminescent western blotting substrate (ThermoFisher Scientific) and developed on Blue Ultra Autorad film (ISC Bioexpress).

Polyclonal rabbit anti-EHD1 (also detects EHD4) and EHD2 were used at 1:2000 dilutions and anti-EHD3 was used at a 1:1000 dilution. Rabbit anti VEGFR2 antibody was purchased from Cell Signaling and was used at a 1:1000 dilution.

D. Gene Knock-Down by Small Interfering RNA (siRNA)

Small interfering RNA (siRNA) oligonucleotides (synthesized by Dharmacon) were transfected into EA.Hy926 cells using Lipofectamine 2000 transfection reagent (ThermoFisher Scientific) following the manufacturer's instructions. Demonstrable knockdown of protein expression was seen within 48 hours of transfection as assessed by western blotting. For EHD2, the ON-TARGET SMART-pool siRNA from Dharmacon GE was used. For EHD1, the following sequence was designed: 5'-gaa aga gat gcc caa tgt c (Dharmacon). As an irrelevant siRNA control, Non-targeting siRNA #5 was used (Dharmacon).

E. Tubulation Assays

EA.Hy926 cells were transfected with siRNAs against EHD 1-4 (along with control siRNA), and 48 hours after transfection cells were passaged and replated into T25 flasks so that they were 80-90% confluent the following day. On the second day, cells were passaged a second time and plated in triplicate in a 96-well plate containing 100% growth factor-reduced Matrigel (BD Biosciences) in the following conditions: serum-free media, media + 50 ng/ml VEGFA165 (Peprotech), and media + 15 μ M sulforaphane (Sigma). After 6 hours, cells were incubated with 6 μ M Calcein AM (Trevigen) for 15 minutes and imaged using the EVOS FL Auto microscope. Image J software was used to analyze images, and tubulation was assessed by average branch point and tubule number.

CHAPTER 3: RESULTS

A. EHD Proteins are differentially expressed in a variety of endothelial beds from multiple organs from wild type mice.

EHD proteins have distinct expression patterns in organs and tissues, and evidence suggests differential expression in endothelial beds. To determine expression patterns of EHD proteins in endothelium, immunofluorescent staining was performed on the following organs harvested from a perfused wild-type mouse: kidney, heart, lungs, liver, and pancreas (Figures 4-16). EHDs were triple stained along with DAPI and either tomato lectin or isolectin b4. Tomato lectin (from *Lycopersicon esculentum*) is a lectin that recognizes N-acetyl glucosamine (GlcNAc) and poly-N-acetyllactosamine and that is used to stain endothelial cells (Porter et al. 1990). Isolectin B4 binds alpha-galactosyl residues (Laitinen 1987).

Kidney

Confirming previous publications, EHD3 is exclusively expressed in the glomerular endothelium and EHD1 and 2 are absent (Figure 5). Interestingly, EHD1 is expressed along with EHD2 in segmental arteries that eventually branch into efferent/afferent arterioles where EHD1 is absent (Figures 4 and 5). This suggests that EHD1 staining becomes undetectable as renal arteries branch into smaller subsets. Finally, EHD1, 2, and 3 are all undetectable in peritubular capillaries (Figure 6), which was previously reported to express EHD4 (George et al. 2011).

Heart

As discussed above, EHD3 is important in cardiomyocyte function, but EHD expression within endothelial beds of the heart has not been examined. Immunofluorescent staining shows high levels of EHD2 expression throughout all

endothelial beds of the heart. The endocardium lines the inside of the heart (Figure 7) and is positive for EHD2 and EHD3 expression. Epicardial arteries penetrate the myocardium and become intramural arteries, which show positive staining for EHD2 and 3. Additionally, EHD1 expression appears detectable at low levels (Figure 8). Intramural arteries eventually branch into arterioles. Veins within the myocardium express EHD2 and 3 while EHD1 is undetectable (Figure 9). Finally, myocardial microvessels, which are highly prevalent in the myocardium, show high levels of EHD2, and no detectable levels of EHD1 and EHD3 (Figure 10).

Lungs

Previous work suggested expression of EHD1 in the endothelium isolated from Tie2GFP mouse lungs (Daneman et al. 2010). Immunofluorescent staining showed positive staining for EHD 1, 2 and 3 in wild type mouse lungs. Pulmonary arteries specifically show strong staining for EHD1 and EHD2. EHD3 is also expressed, but staining is more prominent in the epithelium lining the bronchioles (Figure 11). Additionally, there is positive staining for the three EHDs in pulmonary veins (Figure 12). Finally, EHD 1 and 2 are expressed in the capillaries that align closely with the alveolar epithelium (alveolar capillaries).

Liver

Studies have already established the expression of EHD3 in sinusoidal endothelium within the liver, but nothing is known about other EHD proteins within the sinusoids or other endothelial beds. Both hepatic arteries and portal veins deliver blood to the sinusoids. Immunofluorescent staining showed positive staining for EHD1, EHD2, and EHD3, but EHD1 was undetectable in the portal veins (Figures 13 and 14).

Interestingly not only was EHD3 expressed in the liver sinusoids, but that staining continued into the central veins where blood from the sinusoids is emptied (Figure 15).

Pancreas

Capillaries of the pancreas showed no detectable staining for EHD1, EHD2, and EHD3 within both the islets of Langerhans and acinar cells (Figure 16 and 17). Because of the lack of a reliable EHD4 antibody, it cannot be determined if EHD4 is expressed in place of the other three EHD proteins. Similarly to their expression pattern in other organs, EHD1 and 2 were expressed in the arteries of the pancreas while EHD1 was undetectable in veins (Figure 18). EHD3 was undetectable in both (Figure 18).

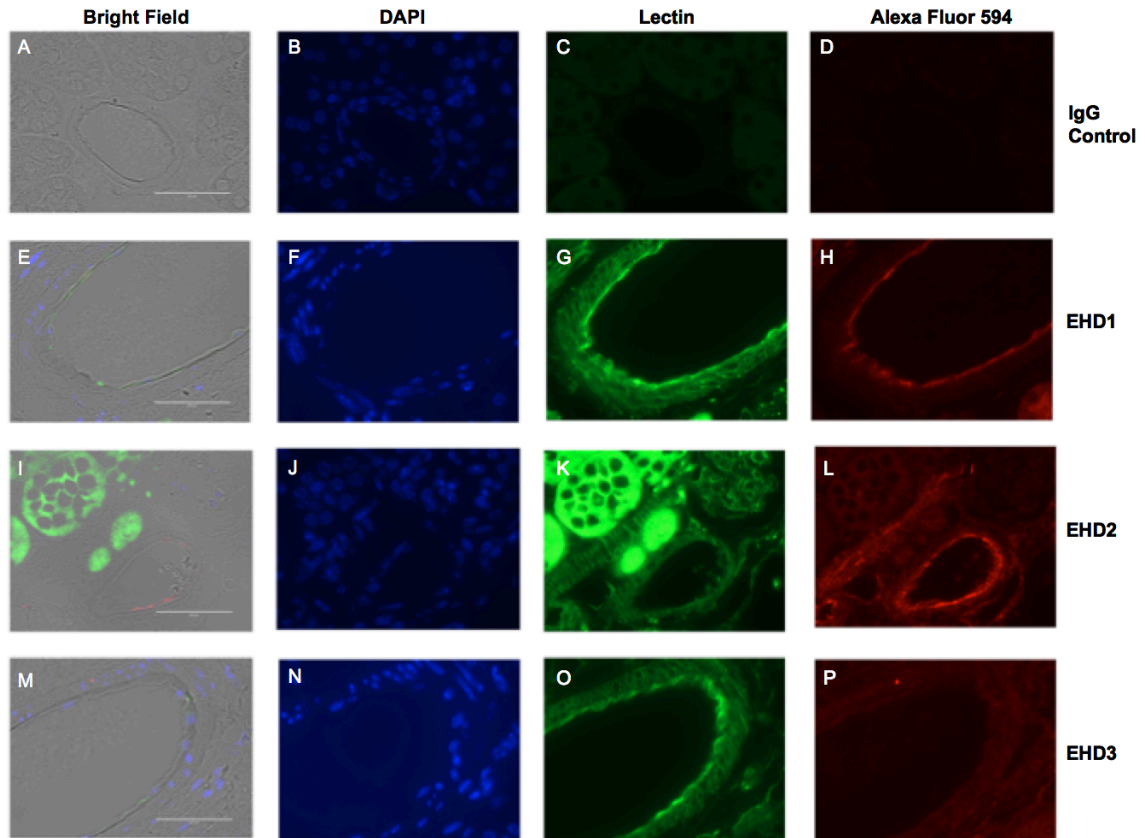


Figure 4: EHD protein expression in segmental arteries of the kidney. (A-P) 5 μ m thick kidney sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. DAPI (blue) stains nuclei (B, F, J, and N), and tomato lectin (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows detectable levels of EHD1 (D) and EHD2 (H) in segmental arteries, but not EHD3 (P). Scale Bar = 50 μ m.

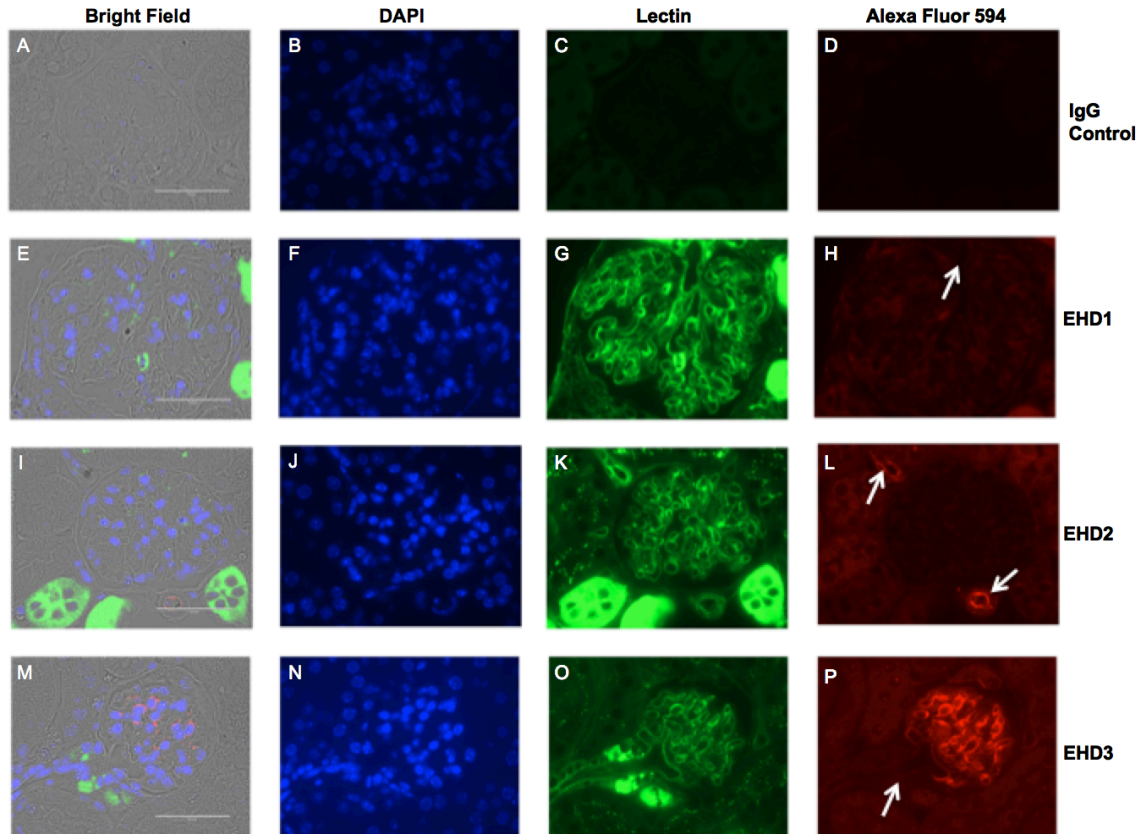


Figure 5: EHD protein expression in glomerular endothelial cells and afferent/efferent arterioles. (A-P) 5 μ m thick kidney sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. DAPI (blue) stains nuclei (B, F, J, and N), and tomato lectin (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows detectable levels of EHD3 in glomerular endothelial cells (P), but not EHD1 and EHD2 (H and L). EHD2 is expressed in afferent/efferent arterioles adjacent to glomeruli (as noted with a white arrow) but EHD1 or 3 are undetectable. Scale Bar = 50 μ m.

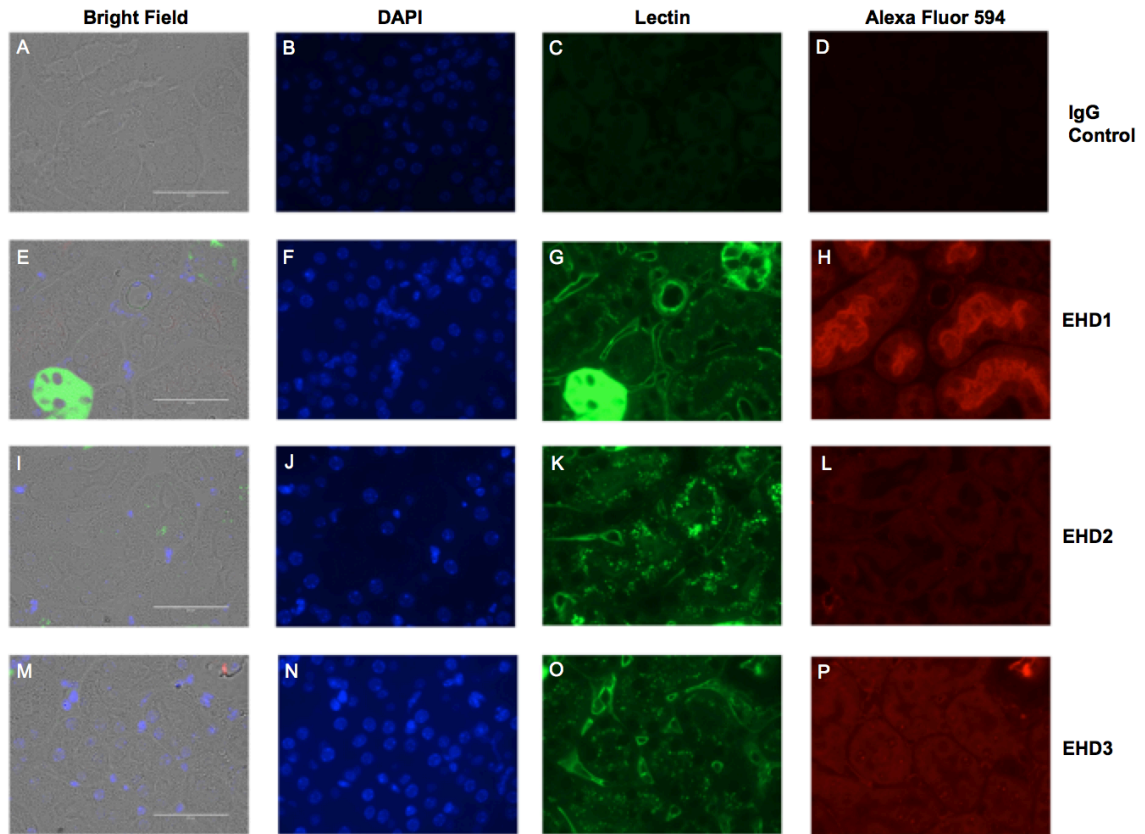


Figure 6: EHD protein expression in peritubular capillaries of the kidney. (A-P) 5 μ m thick kidney sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. DAPI (blue) stains nuclei (B, F, J, and N), and tomato lectin (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows undetectable staining of EHD1, 2, or 3 in peritubular capillaries (D, H, L, and P). EHD1 staining is positive in tubules, though (H). Scale Bar = 50 μ m.

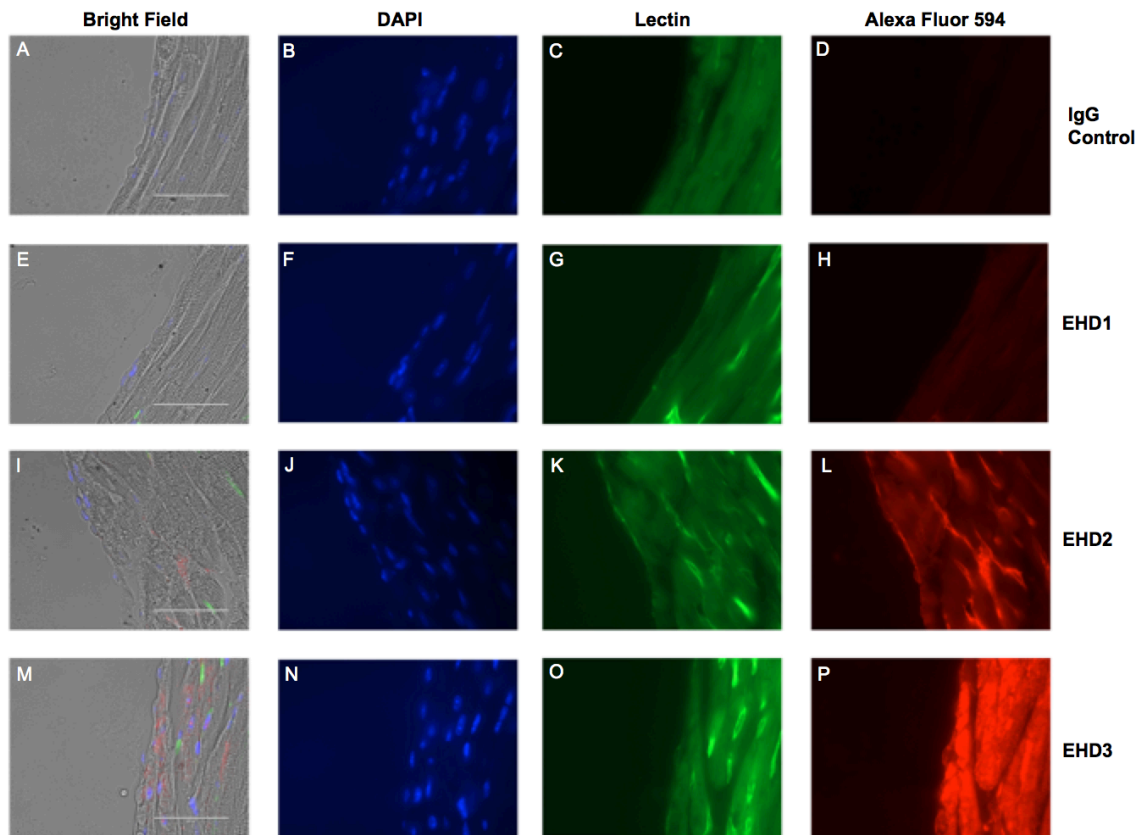


Figure 7: EHD protein expression in the endocardium. (A-P) 5 μ m thick heart sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. DAPI (blue) stains nuclei (B, F, J, and N), and isolectin B4 (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows expression of EHD2 and EHD3 in the endocardium (L and P) and undetectable staining of EHD1 in the endocardium (H). Scale Bar = 50 μ m.

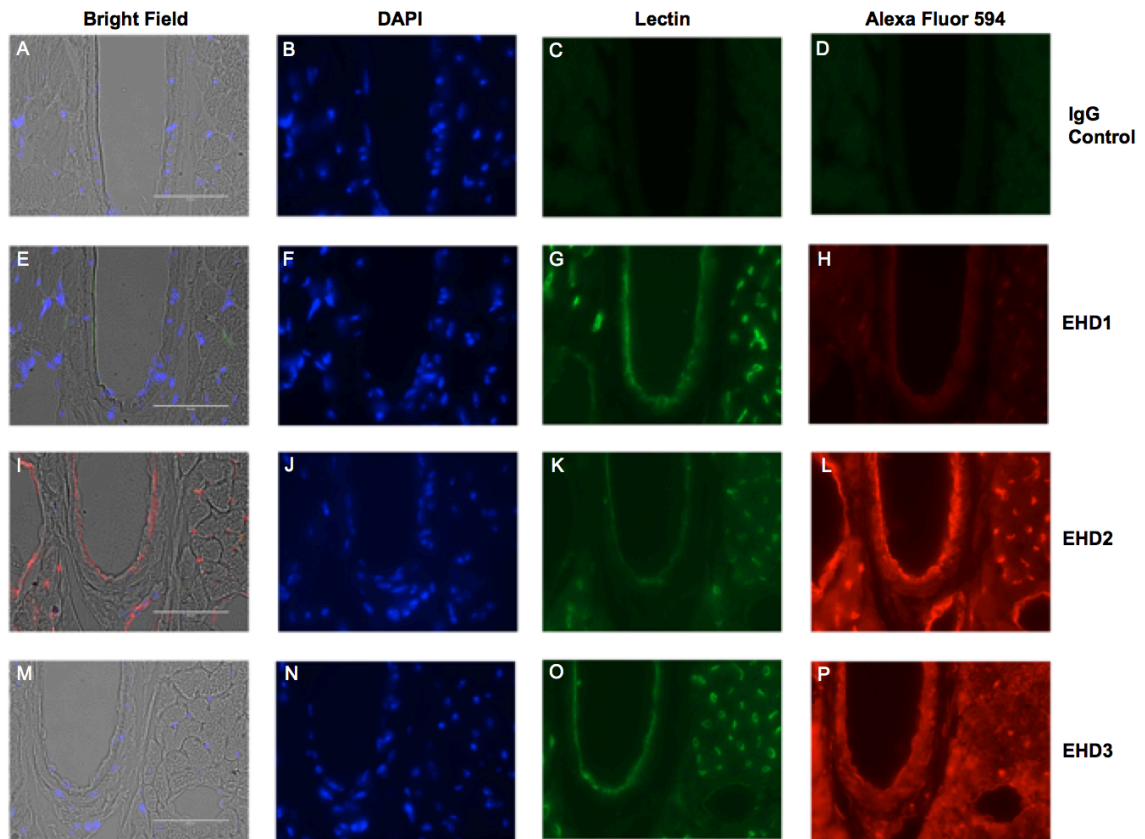


Figure 8: EHD protein expression in intramural arteries of the heart. (A-P) 5 μ m thick heart sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. DAPI (blue) stains nuclei (B, F, J, and N), and isolectin B4 (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows detectable levels of EHD 1, 2, and 3 in intramural arteries (H, L, and P). Scale Bar = 50 μ m.

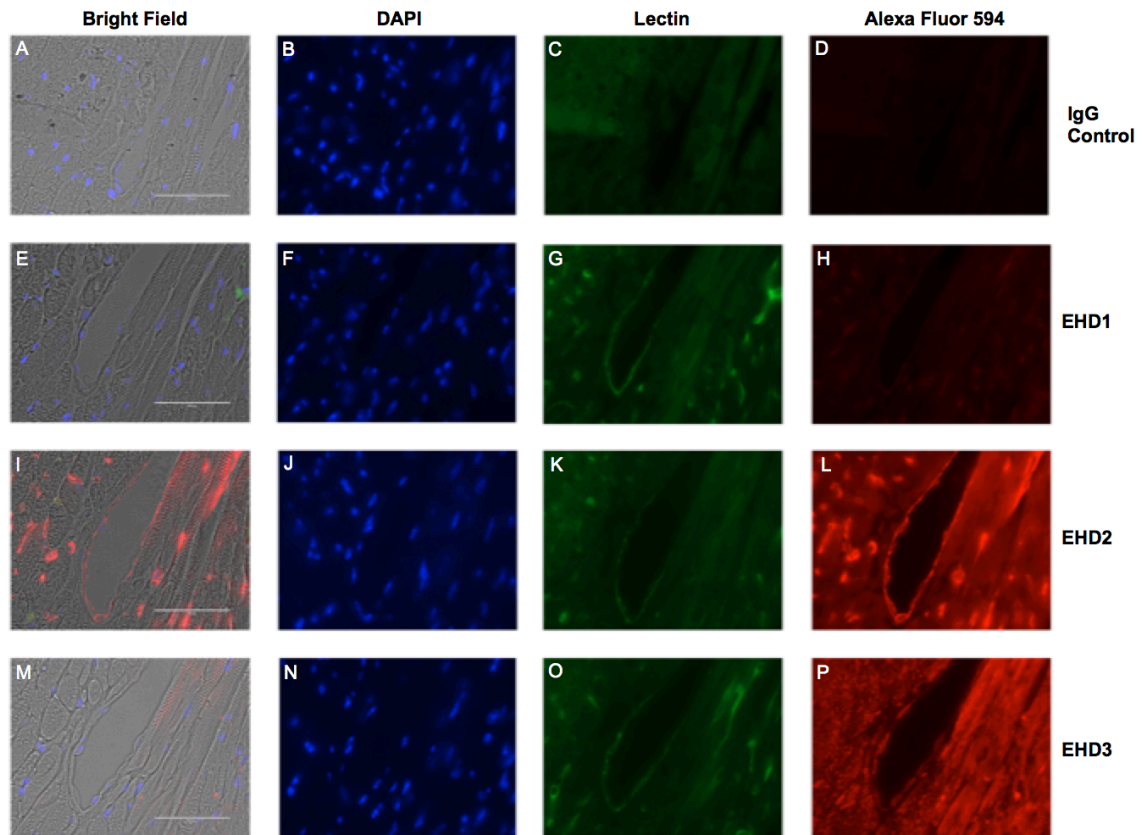


Figure 9: EHD protein expression in veins of the heart. (A-P) 5 μ m thick heart sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. DAPI (blue) stains nuclei (B, F, J, and N), and isolectin B4 (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows no detectable levels of EHD1 (H), but positive staining for EHD2 and EHD3 (L and P). Scale Bar = 50 μ m.

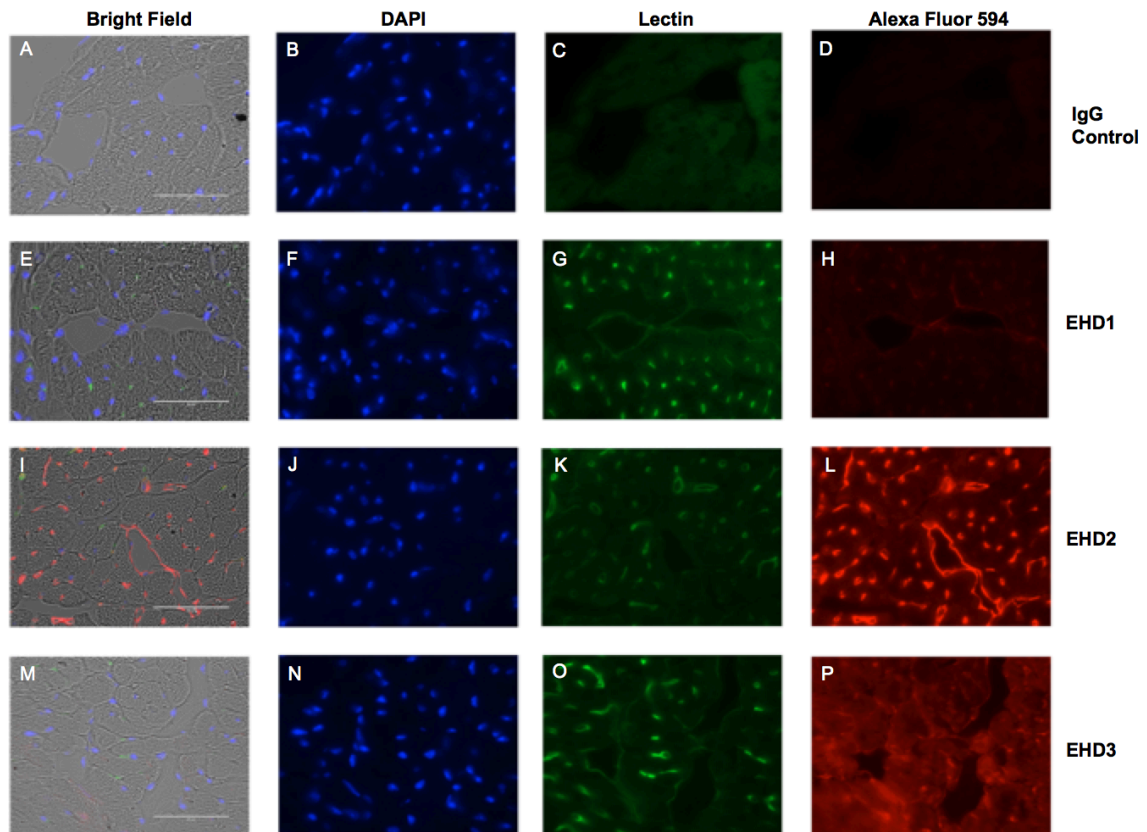


Figure 10: EHD protein expression in myocardial microvessels. (A-P) 5 μm thick heart sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. DAPI (blue) stains nuclei (B, F, J, and N), and isolectin B4 (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows positive staining of EHD2 in the myocardial microvessels (L) and undetectable levels of EHD1 and EHD3 (H and P). Bar = 50 μm .

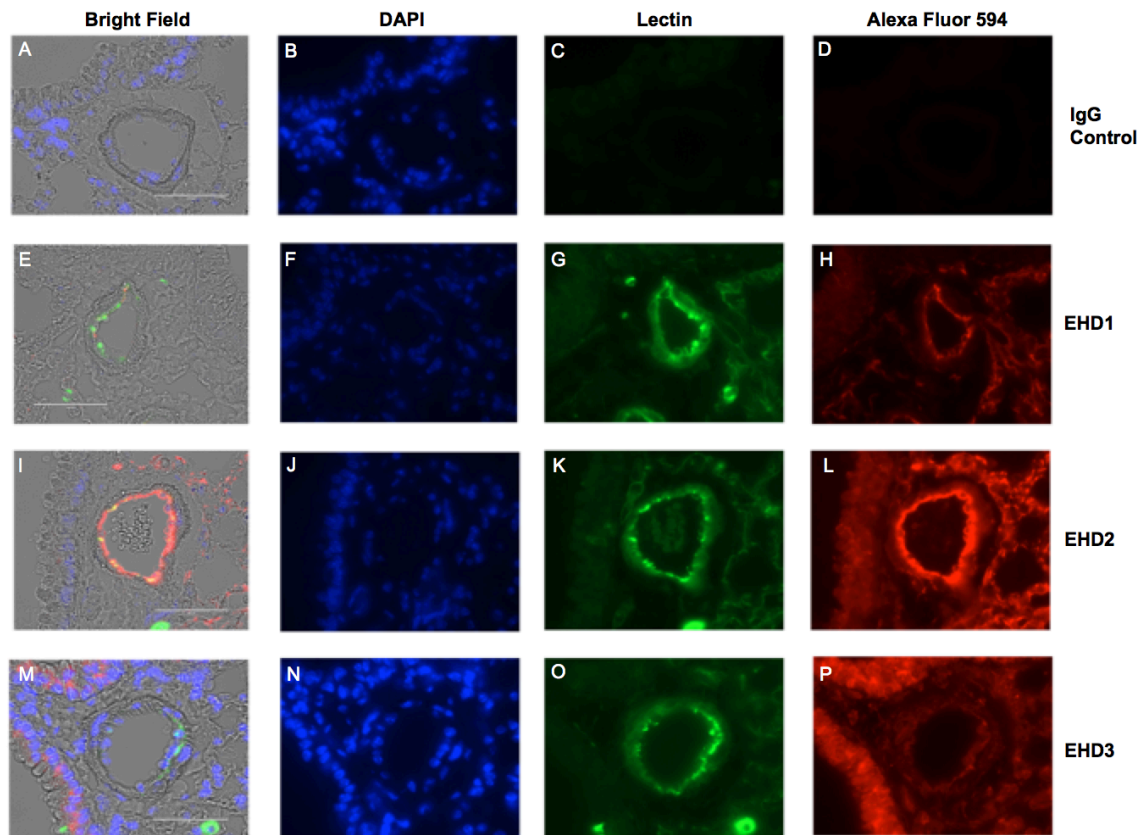


Figure 11: EHD protein expression in pulmonary arteries. (A-P) 5 μ m thick lung sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. DAPI (blue) stains nuclei (B, F, J, and N), and isolectin B4 (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows positive staining for EHD1 and EHD2 (H and L) in pulmonary arteries and low intensity staining for EHD3 (P). Scale Bar = 50 μ m.

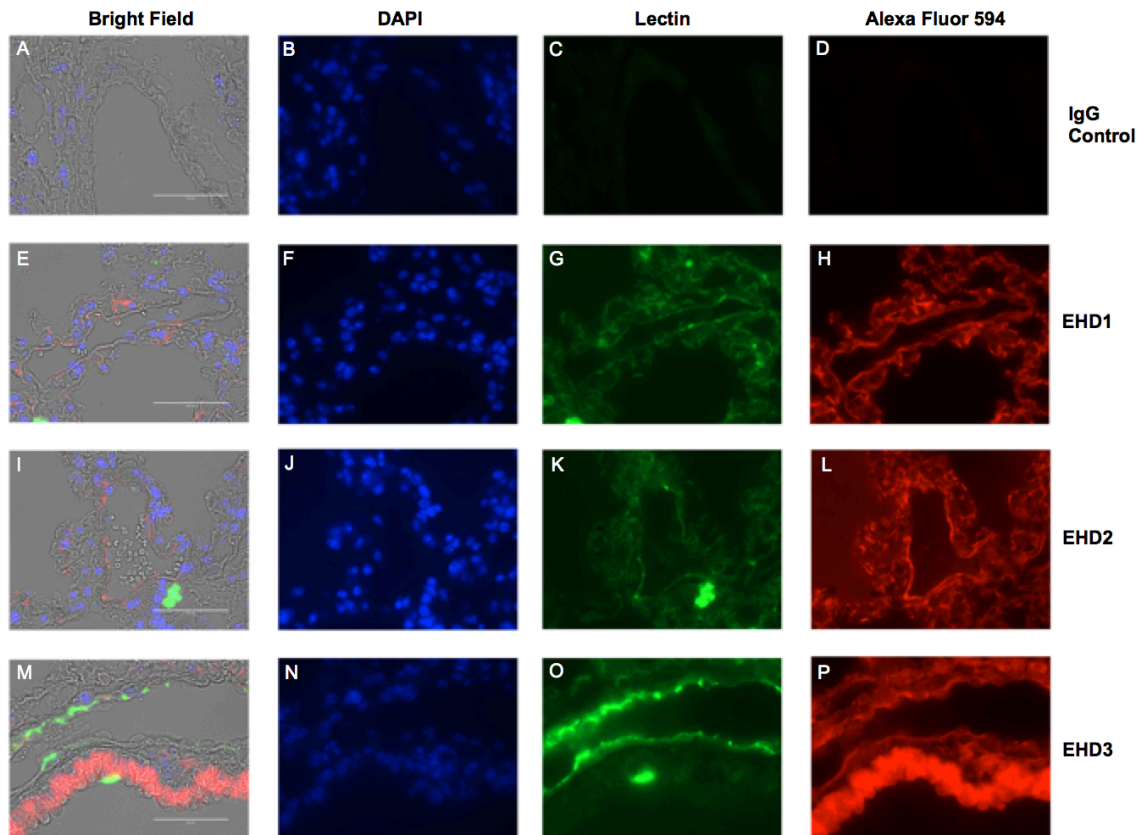


Figure 12: EHD protein expression in pulmonary veins. (A-P) 5 μ m thick lung sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. DAPI (blue) stains nuclei (B, F, J, and N), and isolectin B4 (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows positive staining for EHD1, 2, and 3 in pulmonary veins and EHD1 and 2 in alveolar endothelium (H, L, and P). Scale Bar = 50 μ m.

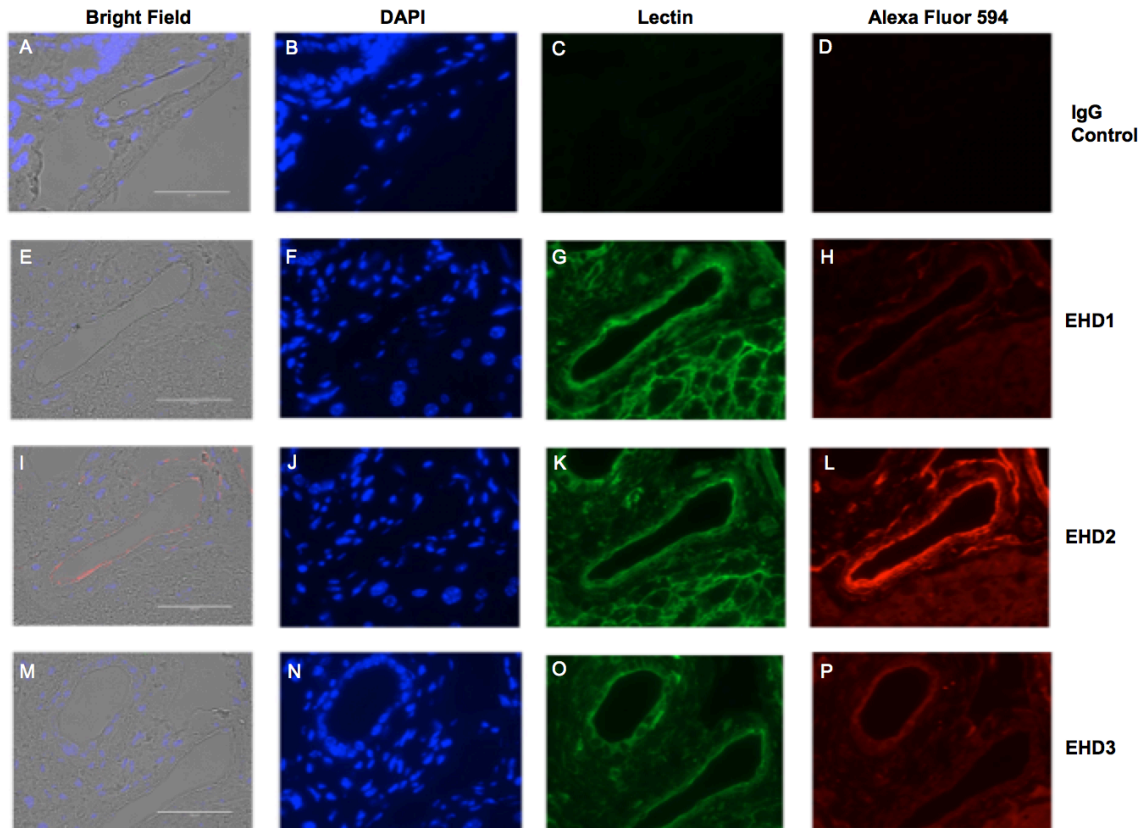


Figure 13: EHD protein expression in hepatic arteries. (A-P) 5 μ m thick liver sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. DAPI (blue) stains nuclei (B, F, J, and N), and tomato lectin (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows detectable levels of EHD1, 2, and 3 in hepatic arteries (H, L, and P). Scale Bar = 50 μ m.

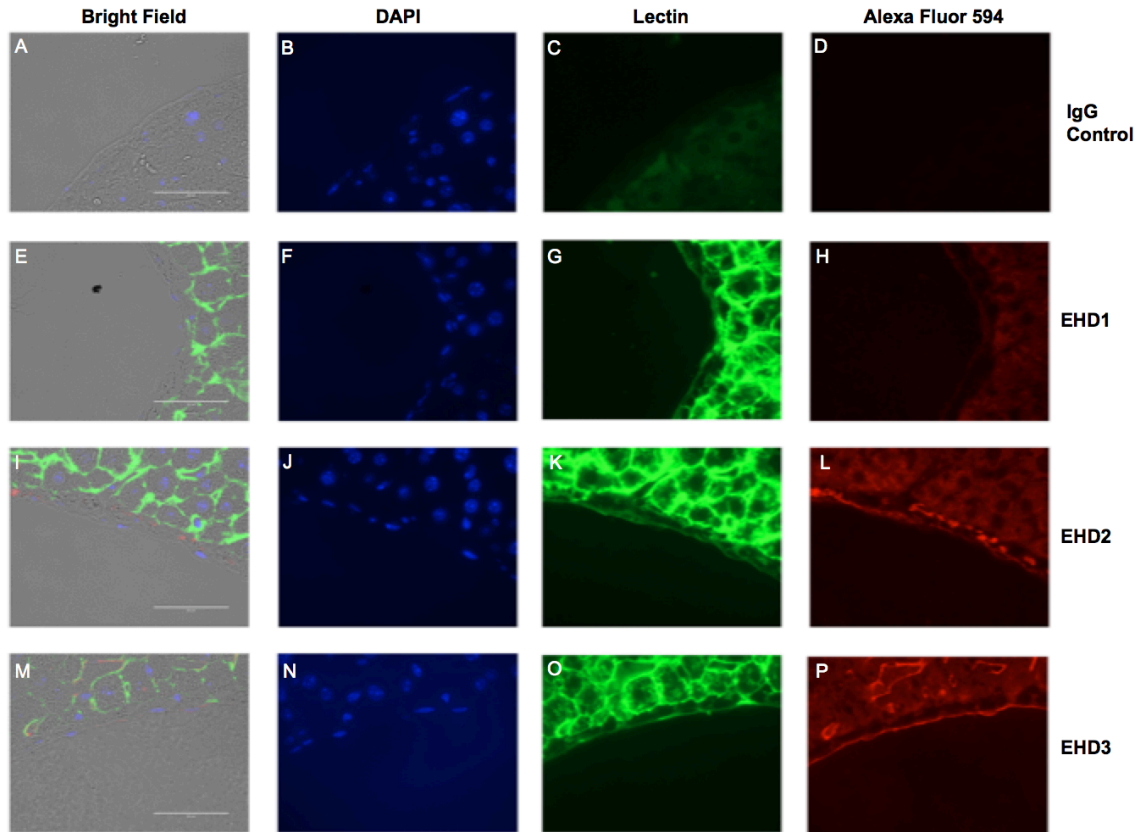


Figure 14: EHD protein expression in hepatic portal veins. (A-P) 5 μ m thick liver sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. DAPI (blue) stains nuclei (B, F, J, and N), and tomato lectin (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows positive staining for EHD2 and EHD3 (L and P) in portal veins, but not EHD1 (H). Scale Bar = 50 μ m.

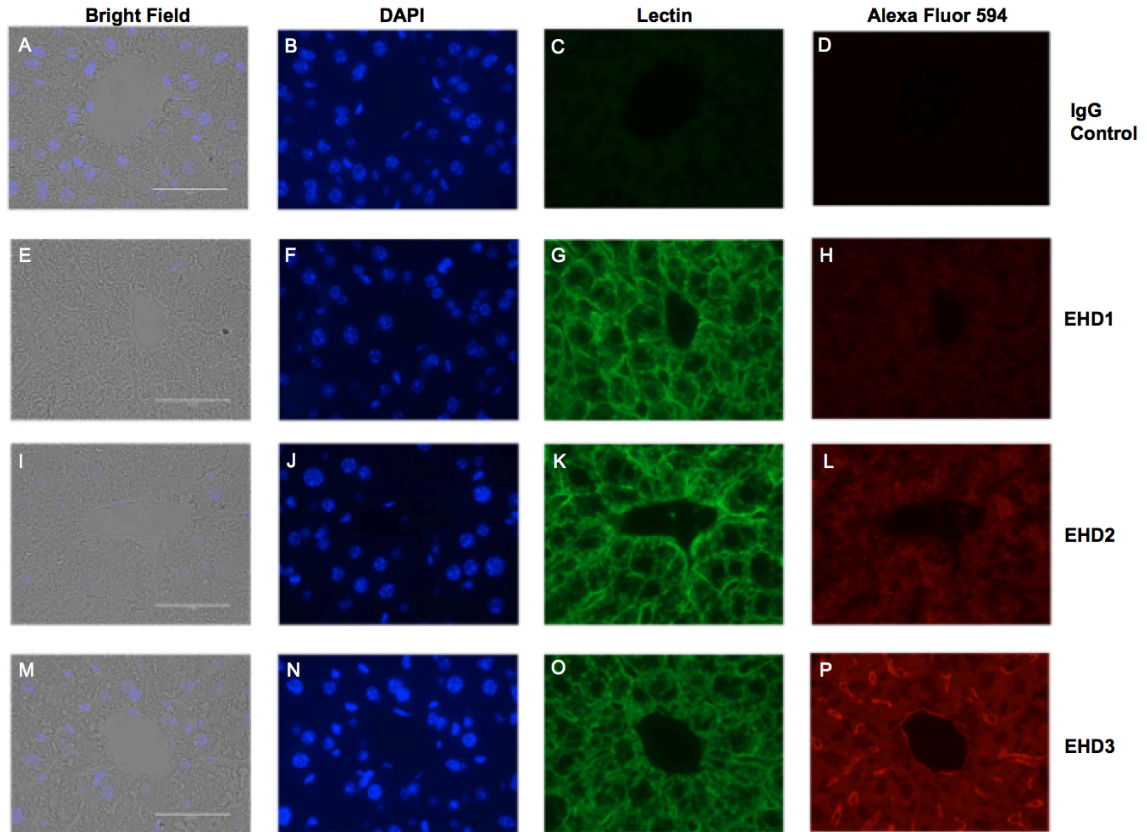


Figure 15: EHD protein expression in liver sinusoids and central veins. (A-P) 5 μm thick liver sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. DAPI (blue) stains nuclei (B, F, J, and N), and tomato lectin (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows detectable levels of EHD3 in central veins and surrounding sinusoids (P), but not EHD1 and EHD2 (H and L). Scale Bar = 50 μm .

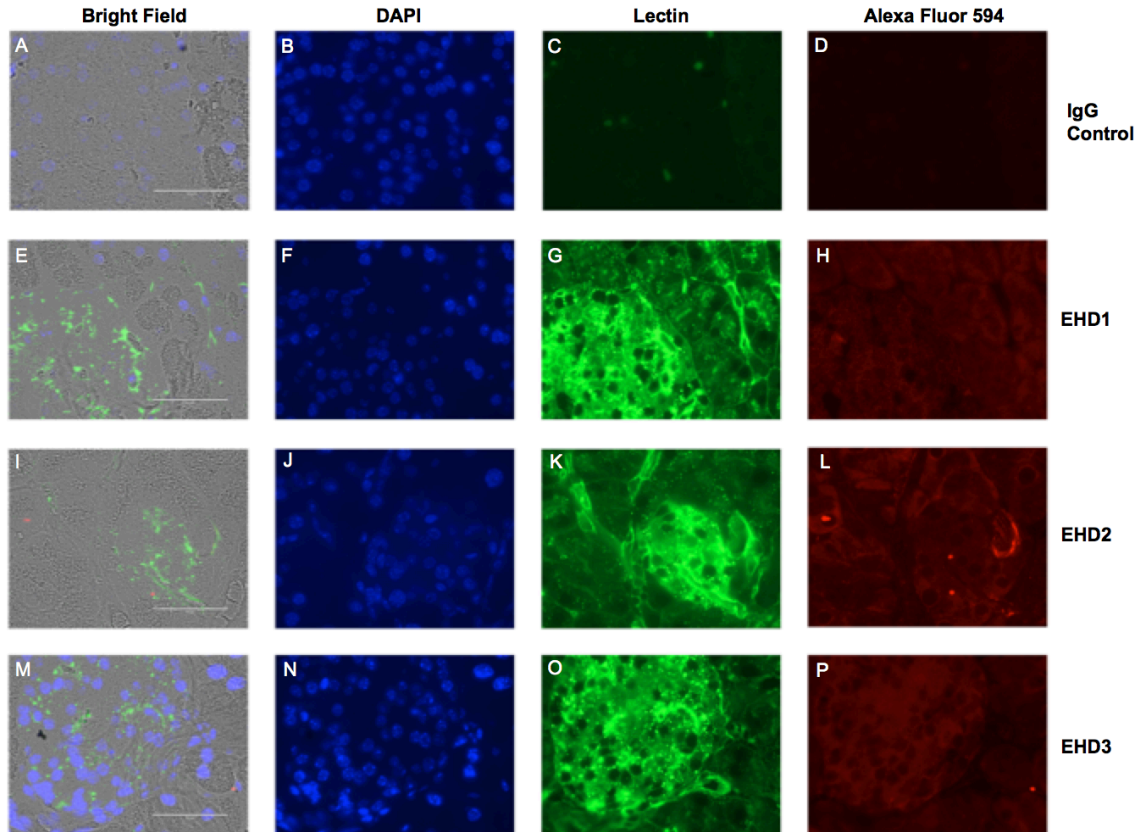


Figure 16: EHD protein expression in capillaries of the islet of Langerhans. (A-P) 5 μ m thick pancreas sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. DAPI (blue) stains nuclei (B, F, J, and N), and tomato lectin (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows no detectable levels of EHD1, 2, or 3 in the capillaries (H, L, and P). Scale Bar = 50 μ m.

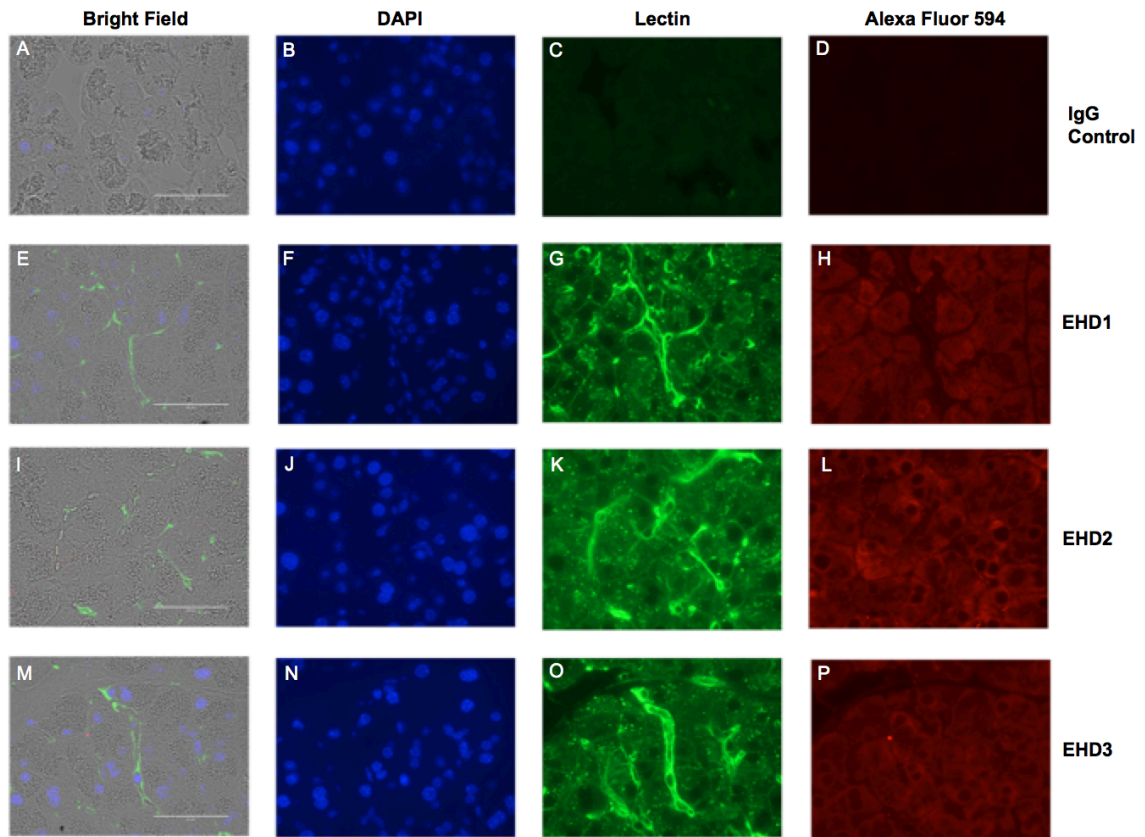


Figure 17: EHD protein expression in capillaries among pancreatic acinar cells. (A-P) 5 μ m thick pancreas sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. DAPI (blue) stains nuclei (B, F, J, and N), and tomato lectin (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows no detectable levels of EHD1, 2, or 3 in the capillaries (H, L, and P). Scale Bar = 50 μ m.

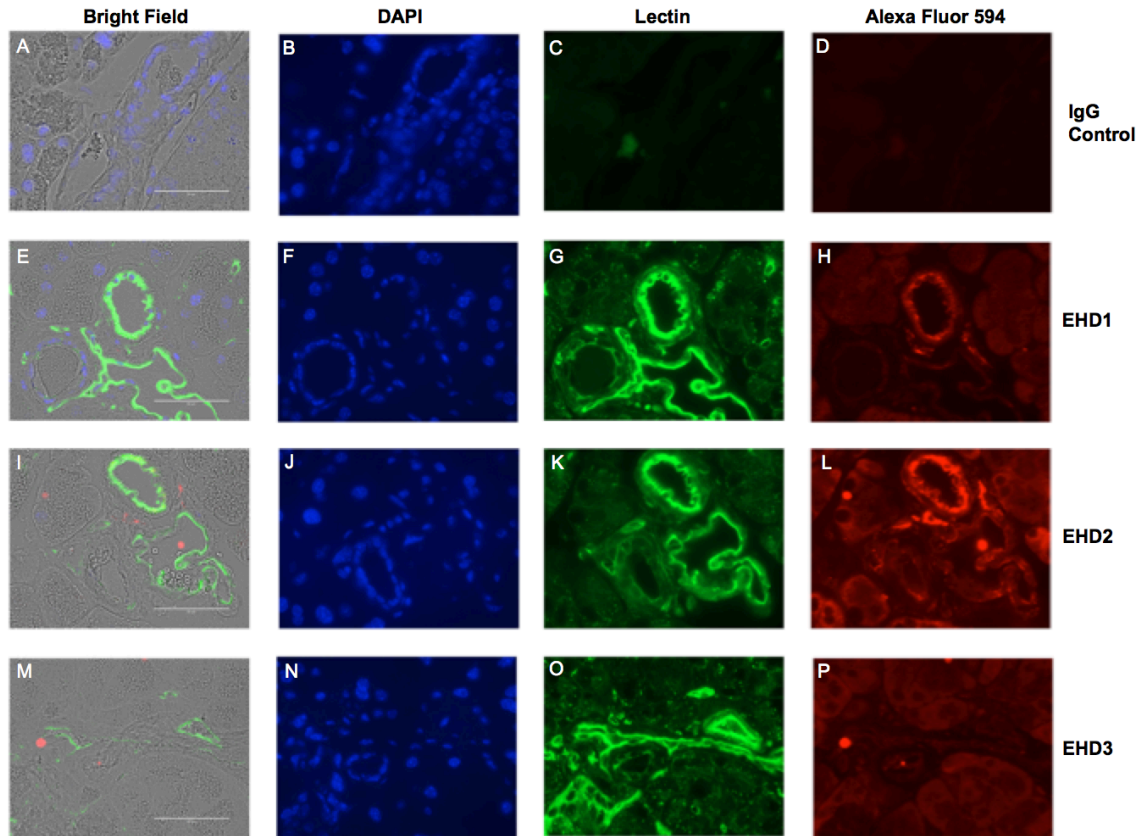


Figure 18: EHD protein expression in arteries and veins of the pancreas. (A-P) 5 μ m thick pancreas sections from perfused wild type mice were stained using antibodies against EHD1, 2, and 3 and fluorescent images were acquired. Tomato lectin (green) labels endothelial cells (C, G, K, and O). EHD staining (red) shows positive staining for EHD1 in arteries, but not veins (H), for EHD2 in both arteries and veins (L), and for neither for EHD3 (P). Scale Bar = 50 μ m.

B. All EHD Proteins are Expressed in Multiple Endothelial Cell Lines

Many *in vitro* studies examining endothelial biology utilize commercially available endothelial cell lines. Because immunofluorescent staining has shown differential expression of EHD proteins within various endothelial beds, multiple cell lines were analyzed via western blot analysis to see if such compartmentalization is lost *in vitro*. Indeed, when comparing several endothelial cell lines all four EHD proteins are expressed, although at varying levels (Figure 19). HUVECs are a primary cell line while EA.Hy926 are HUVECs that have been immortalized by fusing them with a thioguanine-resistant clone of A549 by exposure to polyethylene glycol (PEG) (Campos-Estrada et al. 2015). The HMEC-1 line is isolated from the dermis and immortalized via the SV-40 antigen. Interestingly, within the three commercial cell lines used, the immortalized cell lines expressed higher levels of EHD3 and reduced levels of EHD2. Comparing the commercial lines to a primary cell line isolated from the mouse mammary fat pad (MFP MVEC), EHD levels are again varying. When SV40 antigen is expressed in MFP-MVECs, there is lower expression of EHD4.

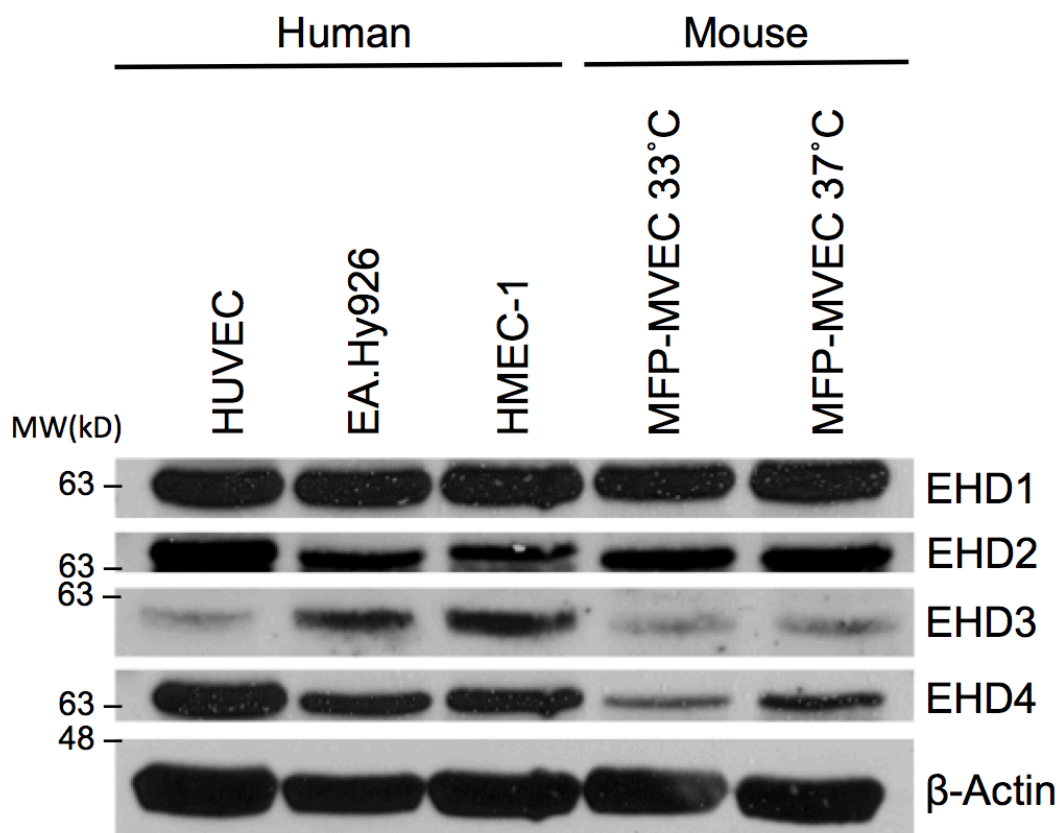


Figure 19: All EHD proteins are expressed in multiple endothelial cell lines. Cell lysates containing 40 μ g of protein were resolved by 7.5% SDS-PAGE and transferred onto PVDF membranes which were subjected to immunoblotting with rabbit-anti-peptide antisera raised against specific EHD proteins. Relative molecular weight markers are indicated in kiloDaltons (kD). β -actin was used as a loading control.

C. siRNA-Mediated Knockdown of EHD2 Does Not Affect VEGFA-Induced Endothelial Cell Tubulation

Continuous endothelium contains the highest level of caveolae and also stained the strongest for EHD2 (Figures 4-18). Since caveolin-1 has been linked to angiogenesis and colocalizes with EHD2 in caveolae in other cell types, siRNA-mediated knockdown of EHD2 was performed to assess the effects on tubulation in Matrigel. *In vitro*, tubulation assays are utilized to test different conditions on the effects of angiogenesis without the expense and difficulties of *in vivo* studies (Arnaoutova & Kleinman 2010). Western blot analysis (Figure 20A) confirmed reduction in EHD2 upon treatment with siRNA. Immunoblotting for remaining EHD proteins showed no increase or decrease in levels upon knockdown of EHD2. Because tubulation was stimulated by VEGFA ligand, protein levels for VEGFR2 were assessed as well. VEGFR2 protein levels appear to remain the same after EHD2 knockdown (Figure 20A). Figure 20B are the 8-bit converted images collected after the tubulation assay was completed. Visually, there appears to be no difference in tubulation between EHD2 knockdown and control, although the EHD2 knockdown tubules may appear thicker. Figure 20C and D show tubule branch point and tubule counts, both means for quantifying tubulation (Arnaoutova & Kleinman 2010). Comparing cells treated with control siRNA to those treated with EHD2 siRNA branch points and tubule counts are approximately the same, and this is true with or without VEGFA stimulation. Taken together, this suggests that siRNA mediated EHD2 knockdown does not affect tubulation *in vitro*.

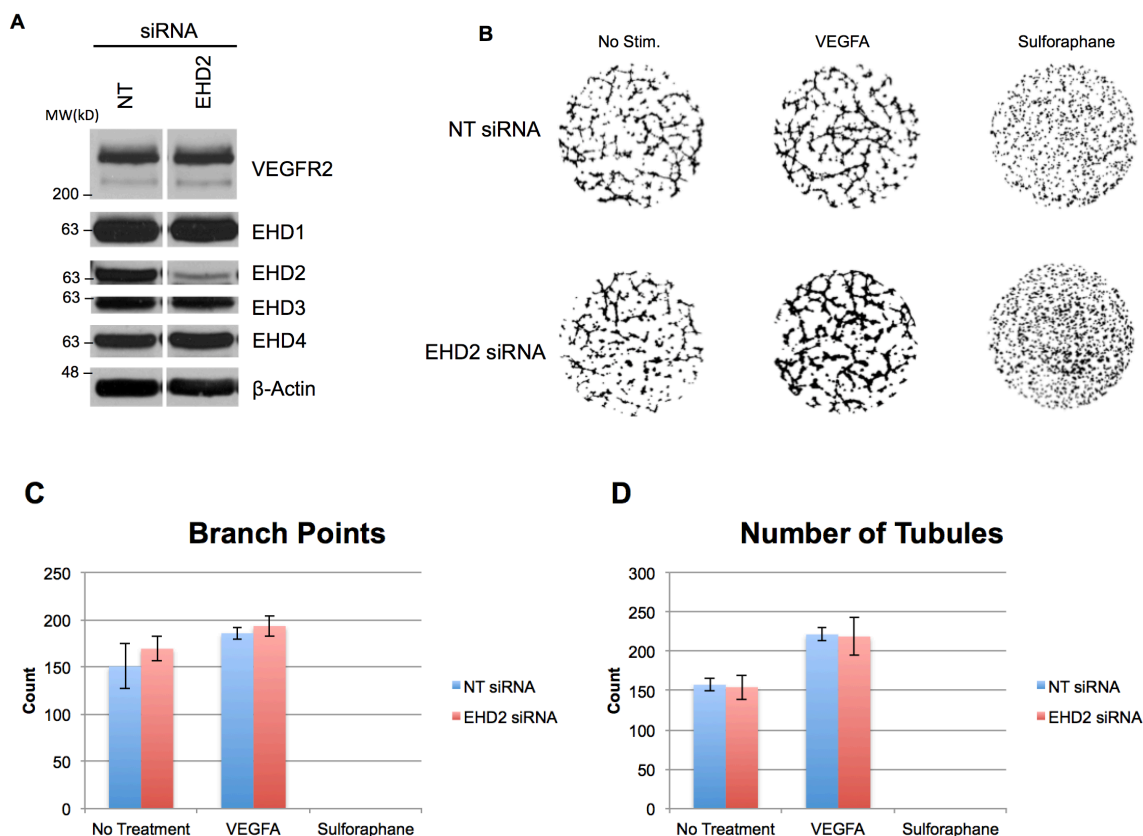


Figure 20: siRNA-mediated knockdown of EHD2 does not affect VEGFA-induced tubulation in EA.Hy926 cells. Cells were treated with non-targeting, EHD1 and EHD2 siRNAs. Western blot analysis (A) confirms reduction in EHD1 and EHD2 as well as an increase in VEGFR2 with EHD1 reduction. B-D shows 8-bit representations of imaged cells and perimeter measurements to represent tubulation. Non-targeting (B) shows a slight increase in tubulation upon VEGFA (50 ng/ml) stimulation compared to media alone. EHD1 (C) and EHD2 (D) reduction showed a marked increase in tubulation in comparison (E). Sulforaphane (15 μ m) was used to inhibit tubulation.

CHAPTER 4: DISCUSSION

The vasculature is implicated in most, if not all, diseases. In order to fully harness the therapeutic potential of the endothelium, research must focus on understanding the heterogeneity of ECs. As mentioned in Table 1, one area in which ECs may vary is the genes/proteins they express. Immunofluorescent staining of multiple organs in wild type mice have shown that three of the four mammalian EHD proteins (EHD1-3) are not equally expressed throughout different endothelial beds, but some patterns seem to be evident.

Immunofluorescent staining of selected organs showed the strongest staining for EHD1 in arteries (except for efferent/afferent arterioles of the kidney) and no detectable staining in veins. When comparing arteries to veins, arteries have higher numbers of tight junctions due to higher blood pressure (Aird 2007b). As previously mentioned, EHD1 was also upregulated in cells cultured to mimic the blood brain barrier, another endothelial bed rich in tight junctions. Tight junctions (TJ) are found in many polarized cells and include occludin, claudins, and junction adhesion molecule (JAM) (González-Mariscal et al. 2003). Tight junctions are dynamic and are trafficked through the cell. Internalization of tight junctions can occur via clathrin-dependent and clathrin-independent pathways, and they then can be sent to the lysosome for degradation, to the trans-golgi network via the retrotransport pathway, or recycled back to the plasma membrane (Chalmers & Whitley 2012). Analysis of eye defects associated with the *Ehd1*-null mouse displayed irregular staining of ZO-1, an occludin TJ, within the lens epithelium (Arya et al. 2015). Taken together, these results suggest that EHD1 may play a role in the trafficking of tight junctions in endothelial cells. Interestingly, EHD1 shows strong expression in the pulmonary arteries and veins of the lung in addition to potential expression in alveolar capillaries. Capillaries of the lungs closely associate with alveoli to

accommodate gas exchange. It is possible that EHD proteins may be important in endothelial maintenance during this process.

In line with previous publications, our results suggest that EHD2 is prevalent in many endothelial beds except for some smaller capillaries, including peritubular capillaries of the kidney and sinusoids and central veins of the liver. As mentioned above, endothelial cells, in general, contain higher levels of caveolae compared to other cell types. EHD2 staining was positive in the endothelial beds that contained the most caveolae, i.e. continuous endothelium. The strongest staining was seen in arteries, which have the most caveolae of all blood vessels. Because ECs are exposed to high levels of shear flow, particularly in the arteries, cell membrane repair is an essential process to maintain the endothelial barrier. Currently, the proposed mechanism for membrane repair upon injury involves calcium influx that is sensed by myoferlin localized to caveolae along with caveolin-1. This induces dynamin-dependent endocytosis and subsequent “patching” of the damaged membrane (Bernatchez et al. 2009; Cipta & Patel 2009). As mentioned above, EHD2 is known to interact with myoferlin and localizes to the site of injury in myoblasts along with dysferlin, another ferlin family member. Knowing that EHD2 localizes to caveolae and interacts with caveolin-1, it is possible that EHD2 plays a role in the membrane repair process in endothelial cells. Due to its localization at the site of injury and its role in myoblast fusion along with myoferlin, both in myoblasts, it further possible that EHD2 may also be involved in the “patching” step of the proposed repair process. In addition to membrane repair, myoferlin has also been implicated in the recycling of vascular endothelial growth factor receptor 2 (VEGFR2) via a caveolae-dependent mediated endocytic pathway (Bernatchez et al. 2007). If EHD2 interacts with myoferlin in ECs, it is possible that EHD2 could also be involved in the trafficking of VEGFR2.

EHD3 expression was consistently low in arteries, but uniquely present in glomerular endothelial cells and liver sinusoids as previously described. Another type of fenestrated endothelial bed includes the presence of diaphragms and includes peritubular capillaries in the kidney and capillaries in the pancreas. As previously mentioned, EHD4 is expressed in peritubular capillaries. Interestingly, no staining of EHD1, 2, or 3 was evident in the pancreatic capillaries. Taken together, EHD4 is predicted to be expressed in the pancreatic microvasculature and immunofluorescent staining will need to be confirmed to perform this.

A potential role for EHD3, and even EHD4, is the regulation of fenestrae development in fenestrated and discontinuous endothelial cells. As previously mentioned, combined *Ehd3* and *Ehd4* deletion in mice causes renal pathology and abnormal localization of VEGFR2 in glomerular ECs. Research has shown that VEGFR2 signaling may play an important role in the formation of fenestrae in both glomerular ECs and liver sinusoids (Satchell & Braet 2009; Carpenter et al. 2005). In brief, it is thought that VEGFR2 activation via VEGFA results in downstream actin rearrangement and recruitment of plasmalemmal vesicle-associated protein-1 (PV-1). PV-1 then assists in the formation of fenestrae and is later removed (Satchell & Braet 2009). EHD3, and perhaps EHD4, may be involved in the trafficking of VEGFR2 during VEGFA signaling specifically involved in fenestrae formation. Endothelial-specific deletion of *Ehd3* and *Ehd4* may be able to shed light on this process.

In addition to characterizing EHD proteins in endothelial beds of different organs, it is now known that EHD proteins are expressed in multiple endothelial cell lines. Unlike the expression patterns seen in tissues, though, there is much broader expression of all four EHD proteins in the four cell lines examined in this study. It is possible that the environment of an endothelial cell greatly influences its EHD protein expression patterns

within specific tissues. It is also possible that isolating more specific endothelial beds, such as glomerular endothelial cells, will show EHD expression similar to what is seen *in vivo*. Additionally, it appears that immortalization procedures affect the expression of some EHD proteins. HMEC-1 and EA.Hy926 have both been immortalized after isolation and both have increased levels of EHD3 and reduced levels of EHD4 compared to HUVEC, which is a primary cell line.

Finally, according to the tubulation experiments, siRNA-mediated reduction in EHD2 did not alter VEGFA-induced tubulation of endothelial cells in Matrigel (Figure 20). Other EHD protein levels appear unaffected by EHD2 reduction, and could be compensating for the reduction in EHD2 (mainly EHD1 or EHD4). Additional *in vitro* studies will need to be performed to explore this further, including assessing the effects of EHD2 knockdown on endothelial cell invasion and migration.

CHAPTER 5: CONCLUSIONS AND FUTURE PROSPECTS

EHD proteins are highly conserved endocytic regulators and have both redundant and unique functions. Their differential expression within endothelial beds has further contributed to the heterogeneous nature of ECs. Despite adding to the complexity of the field of endothelial biology, it is apparent there are patterns in EHD expression and novel functions for these proteins in ECs may soon be identified. To further understand the importance of EHD proteins *in vivo* in the context of vascular research, similar immunostaining or histological analysis should be carried out on current knockout mouse models that are readily available. Currently, no vascular defects have been reported in knockout mice beyond the study performed in mice with combined *Ehd3* and *Ehd4* deletion (George et al. 2011). Due to its high expression in continuous endothelial cells and most blood vessels, an *Ehd2* knockout mouse would be of great interest to study. Currently, there are no publications characterizing *Ehd2* knockout mice. Additionally, an effective, specific EHD4 antibody must be established so that complete characterization can be accomplished. In addition to total knockout mice, endothelial-specific deletion models can also be utilized. Multiple mouse models have been established and could allow for analysis of mice with combined deletions that may be lethal in a whole system knockout model (Gustafsson et al. 2001; Kisanuki et al. 2001; Alva et al. 2006).

In vitro studies have been crucial to help understand the function of EHD proteins in endocytic trafficking. EC lines should likewise be used to better understand EHD proteins in endothelial biology. Another way to induce more accurate representation of an endothelial bed environment *in vitro* could include co-culturing ECs with other cell types, similarly to the study done with bovine brain capillary cells and glial cells. Finally, another way to recreate a physiological environment could include 3-D culture, similarly

to what was done in the tubulation assay, or exposing ECs to shear flow with machines such as a BioFlux.

Caveolae are prominent in ECs and function in many processes. Due to high expression of EHD2 in continuous endothelium, which has the highest levels of caveolae, and the fact that it interacts with caveolin-1, EHD2 is possibly playing a role in some of these functions as well, including membrane repair and angiogenesis. Due to their more compartmentalized expression patterns in fenestrated and discontinuous endothelium, EHD3 and 4 may be important in the development of fenestrae via VEGFR2 recycling. Examining other organs with such beds should include the spleen, bone marrow, endocrine glands, and intestines.

Understanding the role of EHD proteins in endothelial biology will not only provide useful physiological information, but also provide insight into future therapeutic targets. The vascular system has access to every tissue and organ in the body and is a desirable target for drug delivery. One route for drug delivery utilizes the endocytic pathways of endothelial cells to deliver therapeutics to underlying tissues. Understanding EHD proteins in the context of endothelial cells will help to further this area of research and improve upon drug therapies for multiple diseases.

CHAPTER 6: REFERENCES

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